LIPASE-CATALYZED SYNTHESIS OF PHENOLIC LIPIDS IN SOLVENT-FREE MEDIUM USING SELECTED EDIBLE OILS AND PHENOLIC ACIDS

by

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ENZYMATIC SYNTHESIS OF PHENOLIC LIPIDS IN SOLVENT-FREE MEDIUM

This thesis is dedicated to my supportive parents, my husband, my kids, with love.

ABSTRACT

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The enzymatic synthesis of phenolic lipids (PLs) in solvent-free medium (SFM), by transesterification of flaxseed and fish liver oils with selected phenolic acids was investigated, using *Candida antarctica* lipase as the biocatalyst. The enzymatic synthesis of phenolic lipids from flaxseed oil was optimized in terms of water activity, agitation speed, enzyme and phenolic acid concentrations. Increasing the water activity of the flaxseed oil reaction mixture from 0.18 to 0.38 resulted in a significant increase in the bioconversion yield from 62 to 77%. The highest enzymatic activity (178 nmol of PLs/g solid enzyme/min) was obtained with the use of 40 mg of solid enzyme (400 PLU)/mL reaction volume at 150 rpm. Under the optimized conditions there was a significant increase in the proportion of linolenic acid ($C_{18:3}$ *n*-3), which increased from 57% in the flaxseed oil to 75 and 64% in the produced phenolic mono- and diacylglycerols, respectively. The volumetric productivity (Pv) of the transesterification of flaxseed oil and 3,4-dihydroxyphenyl acetic acid (DHPA) in SFM was increased 11-fold as compared to that in organic solvent medium. On the other hand, a bioconversion yield of 61% was obtained for the transesterification of fish liver oil with dihydrocaffeic acid (DHCA). Optimization of the enzymatic synthesis of phenolic lipids in SFM from fish liver oil was carried out, using response surface methodology (RSM), based on a four-factor-five-level central composite rotatable design (CCRD). The optimal conditions for the enzymatic reaction were obtained at 50.0ºC, 20.9 mM phenolic acid, 51.2 mg of solid enzyme (512 PLU)/mL, 160 rpm agitation speed, water activity of 0.5 and 3.45 mg Silica gel/mL. The bioconversion yield obtained under these optimized conditions was 86.5%, which is very close to the predicted value of 84.5%. Hence, the predicted values showed good validation with the experimental ones. The overall results demonstrated that RSM can be applied effectively to optimize lipase-catalyzed synthesis of phenolic lipids in SFM, from fish liver oil and DHCA. Under the optimized conditions, there was a significant increase in the relative proportions of the two highly desirable essential fatty acids, where (EPA, $C_{20:5}$ *n*-3) was increased from 11.5% in the unmodified fish liver oil to 21.2, 20.7, 20.8, 20.1 and 19.8% in dihydrocaffeoylated, 3,4-dihydroxyphenyl acetoylated, caffeoylated, feruloylated and sinapoylated lipids, respectively, whereas (DHA, $C_{22:6}$ *n*-3) increased from 12.0% to 21.4, 19.4, 27.5, 22.1 and 22.0%, respectively. Atmospheric pressure chemical ionization-mass spectrophotometry (APCI-MS) analyses confirmed the formation of six 3,4-dihydroxyphenyl

acetoylated and six dihydrocaffeoylated lipids from the transesterification of flaxseed and fish liver oils in SFM using DHPA and DHCA, respectively, as substrates. Although the synthesized phenolic lipids demonstrated radical scavenging activity, expressed as IC_{50} from 1.6 to 3.7-fold higher than that of its corresponding phenolic acid, it was compared to that of α-tocopherol.

RÉSUMÉ

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La biosynthèse des lipides phénoliques sans solvent, par la transésterification de l'huile de graines de lin (HGL) et l'huile de foie de poisson (HFP) en utilisant comme substrats les acides phénoliques, a été étudiée en utilisant la lipase *Candida antarctica* comme biocatalyseur. L'optimisation de la biosynthèse des lipides phénoliques à partir de l'HGL a été investiguée en considérant l'activité thermodynamique de l'eau, la vitesse d'agitation, la concentration de l'enzyme et l'acide phénolique. Le rendement de la bioconversion a augmenté de 62 à 77% lorsque l'activité thermodynamique de l'eau du mélange réactionnel de l'HGL a augmenté de 0.18 à 0.38. L'activité enzymatique maximale (178 nmol de PLs/g solide enzyme/min) a été obtenue lors de l'utilisation de 40 mg d'enzyme solide (400 PLU/mL de volume réactionnel) à 150 rpm. En utilisant les conditions réactionnelles optimales, la proportion de l'acide linolénique ($C_{18:3}$ *n*-3) a augmenté significativement de 57% dans l'HGL à 75 et 64% dans les produit mono- et diacylglycerols phénoliques, respectivement. La production volumétrique (Pv) de la transésterification de l'HGL et de l'acide dihydroxyphényl acétique (ADHP) en milieu non organique a été 11 fois supérieure à celle obtenue dans le milieu organique. Par ailleurs, le rendement de la bioconversion de 61% a été obtenu lors de la transésterification de l'HFP et de l'acide dihydrocafféique (ADHC). L'optimisation de la synthèse enzymatique des lipides phénoliques a été étudiée en utilisant la méthodologie des surfaces de réponse (MSR), basée sur le factorielle quatre à cinq niveaux sur un plan composite centrale rotatif. Les conditions optimales de la réaction enzymatique ont été déterminées comme suit: 50.0ºC, 20.9 mM d'acide phénolique, 51.2 mg d'enzyme solide (512 PLU)/mL, vitesse de l'agitation 160 rpm, 0.5 de l'activité thermodynamique de l'eau et 3.45 mg de gel Silicate/mL. Le rendement de bioconversion maximum obtenu expérimentalement de 86.5% est très proche de la valeur prédite de 84.5%. Ceci démontre une bonne validation du model. Les résultats, en général, démontrent que la MSR peut être appliquée effectivement pour l'optimisation de la biosynthèse des phénols lipidiques en l'absence de solvant à partir de l'HFP et l'ADHC en utilisant la lipase comme biocatalyseur. Dans les conditions optimales, il y a eu une augmentation significative des proportions relatives des deux acides gras essentiels désirés. L'EPA ($C_{20:5}$ *n*-3) dans l'HFP modifié a augmenté de 11.5% à 21.2, 20.7, 20.8, 20.1 et 19.8% dans les lipides dihydrocaffeoylates, 3,4-dihydroxyphenyl acetoylates, caffeoylates, feruloylates et sinapoylates,

respectivement, alors que (DHA, $C_{22:6}$ *n*-3) a augmenté de 12.0% à 21.4, 19.4, 27.5, 22.1 et 22.0%, respectivement. Les analyses de l'ionisation chimique à pression atmosphérique-spectroscopie de masse (APCI-MS) confirment la formation de six 3,4-dihydroxyphényl acetoylates et de six lipides dihydrocaffeoylates, à partir de la transésterification de l'HGL et de l'HFP avec l'ADHP et l'ADHC, respectivement. Les lipides phenoliques synthetisés ont demontré un pouvoir radicalaire, exprimé par le IC_{50}, de 1.6 à 3.7-fois supérieure à celui des acides phénoliques correspondants, mais il était comparable à celui de α-tocophérol.

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CLAIMS OF ORIGINAL RESEARCH

1. This is the first study on lipase-catalyzed transesterification of edible oils as flaxseed and fish liver oils with selected phenolic acids in solvent-free media.

2. Optimization of lipase-catalyzed transesterification of flaxseed oil with 3,4-dihydroxyphenyl acetic acid in solvent-free media was carried out for the first time.

3. The transesterification of fish liver oil with dihydrocaffeic acid in solvent-free medium was optimized for the first time using response surface methodology.

4. The structural characterization and antioxidant properties of the synthesized phenolic lipids obtained from selected phenolic acids with flaxseed and fish liver oils in solvent-free medium, were carried out for the first time.

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LIST OF ABBREVIATIONS

n-3: Omega-3

n-6: Omega-6

n-9: Omega-9

AA: Arachidonic acid

AOCS: American Oil Chemists' Society

AOT: Sodium bis-2-ethylhexyl sulfosuccinate

ANOVA: Analysis of variance

APCI-MS: Atmospheric pressure chemical ionization mass spectrometry

a_w: Water activity

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoulene

CCRD: Central composite rotatable design

CTAB: Hexadecyltrimethylammonium bromide

CVD: Cardiovascular diseases

DHA: Docosahexaenoic acid

DHCA: Dihydrocaffeic acid

DHPA: 3,4-dihydroxyphenyl acetic acid

DAG: Diacylglycerol

$DPPH^{\bullet}$: 2, 2-diphenyl picryl hydrazyl

EPA: Eicosapentaenoic acid

ESI-MS: Electrospray ionization mass spectrometry

FAs: Fatty acids

FFAs: Free fatty acids

FID: Flame ionization detection

GLC: Gas-liquid chromatography

H_2O: Water

HDL: High density lipoprotein

HPLC: High-performance liquid chromatography

LA: Linoleic acid

LCFA: Long-chain fatty acid

LC-MS: Liquid chromatography-mass spectrometry

LC PUFAs: Long-chain polyunsaturated fatty acids

LDL: Low density lipoprotein

LNA: Linolenic acid

MDA: Malonaldehyde

MCFA: Medium chain fatty acid

MAG: Monoacylglycerol

P_E: Enzymatic productivity

PG: *n*-Propyl gallate

PLs: Phenolic lipids

PLU: Propyl laurate units

P_M: Mass productivity

P_V: Volumetric productivity

PUFAs: Polyunsaturated fatty acids

PV: Peroxide value

RSM: Response surface methodology

SCL: Surfactant-coated lipase

SFM: Solvent-free medium

SLs: Structured lipids

Span 65: Sorbitan tristearate

STD: Standard deviation

TAG: Triacylglycerol

TBARS: Thiobarbituric acid reactive substances

TBHQ: *tert*-Butylhydroquinone

TLC: Thin-layer chromatography

UV: Ultraviolet

CHAPTER I

INTRODUCTION

There is a growing interest in the development of nutraceuticals as food supplements as well as natural bio-ingredients to fulfill the high consumer's demand for health-promoting food products. The numerous health benefits of the ω-3 polyunsaturated fatty acids (PUFAs) have been widely recognized in the modulation of risk of a variety of diseases (Roche, 1999; Lee *et al*., 2006; Von Schacky and Harris, 2007; Riediger *et al*., 2009). The incorporation of selected PUFAs into the diet is therefore of great importance.

Flaxseed oil is a good source of the essential fatty acids, such as linoleic acid (LA, $C_{18:2}$ *n*-6) and linolenic acid (LNA, $C_{18:3}$ *n*-3), required in the diet for normal health (Dobson, 2002; Russo, 2009). These fatty acids, being precursors of eicosanoids, have been linked to a wide range of physiological functions and hormonal activities (Roche, 1999; Halsted, 2003; Russo, 2009). The level of LNA in traditional flaxseed oil is generally higher than 50% of the total fatty acids (Daun *et al*., 2003), while the amount of LA is around 14%. LNA was reported to be the principal precursor for ω-3 PUFAs of which eicosapentaenoic (EPA, $C_{20:5}$ *n*-3) and docosahexaenoic acid (DHA, $C_{22:6}$ *n*-3) are the most prevalent, and known to have beneficial effects in health and in the control of some chronic diseases (Tapiero *et al*., 2002; Larsson *et al*., 2004; Jung *et al*., 2008; Riediger *et al*., 2009).

On the other hand, fish oil is the main dietary source of the ω-3 long-chain PUFAs, EPA and DHA. Beneficial health effects of ω-3 PUFAs, especially EPA and DHA are well demonstrated, mainly in the prevention of cardiovascular diseases, autoimmune diseases, rheumatoid arthritis and cancers (Calder, 2001; Larsson *et al*., 2004; Riediger *et al*., 2009). Fish oils is the major source of EPA and DHA with menhaden, sardines, anchovy, herring and cod liver being the major commercial sources (Yamaguchi *et al*., 2004). Consequently, there is a great interest in the utilization of edible oils, such as flaxseed and fish oils that contain ω-3 PUFAs as nutraceutical supplements.

Although PUFAs are found in vegetable and marine oils, the formulation of nutraceuticals with ω-3 PUFAs cannot be done without simultaneous incorporation of an appropriate antioxidant,

since they are prone to oxidative deterioration, which could lead to the formation of off-flavors, reducing the nutritive value of food and producing potentially harmful radical products (Ito *et al*., 1986; Addis and Warner, 1991). Lipid oxidation also causes aging, heart diseases, mutagenesis and carcinogensis (Barlow, 1990). In order to suppress lipid oxidation for maintaining the safety and effectiveness of food, the addition of a suitable antioxidant is required.

However, synthetic antioxidants which are believed to be potentially carcinogenic (Shahidi and Naczk, 1995a) are currently being replaced by natural antioxidants from plant sources (Torres de Pinedo *et al.,* 2007). In addition to their antioxidant properties, phenolic compounds have also other vital beneficial properties in biological systems (Silva *et al*., 2000). Nevertheless, the low solubility of phenolic acids in non-polar media reduces their effectiveness in fat and oil systems (Stamatis *et al*., 2001). The incorporation of phenolic acids into triacylglycerols (TAGs) by lipase-catalyzed transesterification could potentially result in novel structured phenolic lipids (Akoh *et al.,* 1998), with enhanced anti-oxidative, improved solubility and functional properties (Stamatis *et al*., 2001; Sabally *et al*., 2007).

In the last decade, the production of new lipids with functional properties, i.e.; "structured lipids" (SLs) or "tailor-made fats" has greatly increased. SLs consist of TAGs in which the composition and the distribution of fatty acids (FAs) at the glycerol backbone are modified (Osborn and Akoh, 2002). Lipid modification strategies for the production of functional fats and oils, include chemically or lipase-catalyzed transesterification reactions of oils. The modified fats, with novel properties are considered important in medical, nutraceutical and food applications (Osório *et al.,* 2009). SLs obtained through chemical methods require high temperatures and pressure, however, the reaction lacks specificity and offers little or no control over the positional distribution of FAs in the final product (Willis and Marangoni, 1999). Enzymes such as lipases can be used to synthesize SLs under milder conditions, which are suitable for heat sensitive compounds (Akoh *et al.,* 1998), the specificity and selectivity of lipases could be used to generate specific SLs, which cannot be produced by chemical catalysts. A lot of research has been conducted using enzymes to produce SLs with specific FAs to obtain products for specific nutritional or health purposes (Softly *et al*., 1994; Akoh *et al*., 1998; Hita *et al*., 2009; Osório *et al.,* 2009; Chakraborty *et al*., 2010).

Phenolic lipids are SLs that possess a polyunsaturated hydrocarbon chain and an aromatic ring bearing one or more hydroxyl or methoxyl substitutes, attached via an ester bond (Lue *et al.,* 2005). These novel structured molecules could offer the numerous combined beneficial properties of both PUFAs and phenolic acids, with enhanced anti-oxidative and functional properties (Stamatis *et al*., 2001; Sabally *et al*., 2007).

Research work, carried out recently in our laboratory has succeeded in the enzymatic synthesis of structured phenolic lipids in organic solvent media (OSM), including lipase-catalyzed esterification of fatty alcohols and phenolic acids (Lue *et al*., 2005), and the transesterification of phenolic acids with acylglycerol models (Sabally *et al.,* 2006a; Safari *et al*., 2006) as well as edible oils, including flaxseed and fish liver oils (Sabally *et al.,* 2006b; Sabally *et al.,* 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). However, a major drawback limiting the enzymatic synthesis of phenolic lipids in OSM at a large scale is the low volumetric productivity. In addition, the use of some organic solvents may limit the acceptability of nutraceuticals and food ingredients (Martinez *et al*., 2004). The development of an efficient and cost effective process for the enzymatic synthesis of these structured phenolic lipids in a large scale is of utmost importance. One of the most promising novel approaches consists of using solvent-free medium (SFM), which should allow the use of a smaller reaction volume, higher substrate concentrations and avoid the process of solvent-recovery as compared to OSM. Downstream processing is easier as fewer purification steps are required providing significant cost savings, as well as toxic organic solvents are completely avoided (clean conversions), and an increase in the volumetric productivity can be achieved (Dossat *et al.,* 2002a; Fregolente *et al*., 2008). In fact, just to name a few examples, lipases have been recently used in SFM, either in direct esterifications or in transesterifications, for the synthesis of esters and glycerides (Guyot *et al.,* 1997; Fregolente *et al*., 2008; Chaibakhsh *et al*., 2009; Xin *et al.,* 2009). Hence, the development of a bioprocess for the lipase-catalyzed synthesis of phenolic lipids in SFM is of major interest.

The present work was aimed at the lipase-catalyzed synthesis of phenolic lipids using selected edible oils and phenolic acids in solvent-free medium. The synthesized phenolic lipids could be used as nutraceuticals products. The specific objectives were:

(1) To investigate lipase-catalyzed transesterification of flaxseed oil, with selected phenolic acids in solvent-free medium.

(2) To optimize the enzymatic synthesis of phenolic lipids from flaxseed oil with a selected phenolic acid, in solvent-free medium.

(3) To carry out the lipase-catalyzed transesterification of fish liver oil, with selected phenolic acids in solvent-free medium.

(4) To optimize the enzymatic synthesis of phenolic lipids from fish liver oil with a selected phenolic acid in solvent-free medium, using response surface methodology.

(5) To determine the structural characteristics and the antioxidant activity of the synthesized phenolic lipids.

This thesis consists of six chapters. Chapter one provides a general introduction. Whereas Chapter two covers the literature review, related topics and concept used to undertake the research work. Chapter three describes the development of a model reaction conditions for the enzymatic synthesis of phenolic lipids from flaxseed oil and selected phenolic acids in SFM, which was further investigated and optimized, with the use of 3,4-dihydroxyphenyl acetic acid as reported in Chapter four. Chapter five describes the structuring of fish liver oil, with selected phenolic acids, for the enzymatic synthesis of selected phenolic lipids in SFM. Finally, Chapter six describes the optimization of the enzymatic synthesis of phenolic lipids from fish liver oil and dihydrocaffeic acid in SFM, using response surface methodology.

CHAPTER II

LITERATURE REVIEW

2.1. Introduction

Selected edible oils, such as flaxseed and fish liver oils contain polyunsaturated fatty acids (PUFAs), which have important functional and physiological roles in human diets (Roche, 1999). PUFAs are widely recognized in the modulation of risk of a variety of diseases including cardiovascular, immunological, cancer, visual impairment and memory loss (Riediger *et al*., 2009). However, due to their high content of PUFAs, these oils are susceptible to oxidation producing off-flavors and potentially harmful radical products (Ito *et al*., 1986; Addis and Warner, 1991). The autooxidation process could be minimized by the use of suitable antioxidants, such as phenolic compounds. Thus, structuring of these oils with phenolic acids could generate novel compounds with the combined functional and health benefits of both molecules.

2.2. Lipids

2.2.1. Definition and Classification

Lipids are hydrocarbon-containing organic compounds. They are characterized by their insolubility in water and their solubility in non-polar solvents. Lipids can be classified into three main classes, which are simple, derived or complex lipids based on the structure (Christie, 1982). Those containing esters of fatty acids with acylglycerols including mono-, di- and triacylgycerols, are called simple lipids. Complex lipids consist of three or more components such as glycerophospholipids. While fatty acids and alcohols are generally considered as derived lipids, since they can be obtained from components of simple and complex lipids, fatty alcohols are however, usually derived from their corresponding acids (Gurr and Harwood, 2002).

Human's best source of compact chemical energy comes from the dietary lipids; which provide twice the caloric value of an equivalent weight of sugar. Lipids are also vital to the structure and biological function of cells as well as being carriers of the nutritionally essential fat-soluble vitamins. Dietary lipids are mostly obtained from the structural and storage lipids of animals and plants and usually consist of more than 95% triacylglycerols (TAGs). The most important aspect

of dietary lipids is their content of different types of fatty acids, such as saturated, monounsaturated and PUFAs, which are involved in many vital biological activities, such as immune and inflammatory processes (Larsson *et al.,* 2004; Russo, 2009).

2.2.2. Major Sources of Polyunsaturated Fatty Acids

Fatty acids (FAs) are grouped according to the number of carbons, the number of double bonds and the position of the first double bond in the chain. PUFAs are FAs with at least eighteen carbons and between two and six double bonds (Whelan, 1997). Three distinct groups categorize unsaturated fatty acids, *n*-3, *n*-6 and *n*-9. Examples of unsaturated fatty acids belonging to each group, include the long-chain monounsaturated oleic acid ($C_{18:1}$, *n*-9), the double bonded polyunsaturated linoleic acid ($C_{18:2}$, *n*-6) and linolenic acid ($C_{18:3}$, *n*-3) containing three double bonds. Selected PUFAs of dietary significance are linoleic acid (LA), linolenic acid (LNA), arachidonic acid (AA, $C_{20:4,}$ *n*-6), eicosapentaenoic acid (EPA, $C_{20:5,}$ *n*-3) and docosahexaenoic acid (DHA, $C_{22:6,}$ *n*-3) (Deckere *et al*., 1998; Russo, 2009). Current research interest centers on PUFAs, which are involved in vital biological activities, such as inflammatory, immune and cancer processes (Simopoulos, 1991; Larsson *et al.,* 2004; Simopoulos, 2008; Russo, 2009).

There are two major classes of PUFAs, ω-3 (*n*-3) and ω-6 (*n*-6). The *n*-3 has their first double bond at the 3rd carbon atom from the methyl end of the chain, while the *n*-6 has the first double bond located on 6th carbon atom from the methyl end (Deckere *et al*., 1998). LA ($C_{18:2}$, *n*-6) and LNA ($C_{18:3}$, *n*-3) are essential fatty acids that cannot be synthesized by precursors in the body, yet possess beneficial health properties and generally can be obtained through the diet from vegetable oils. While the long chain PUFAs (EPA, $C_{20:5}$, *n*-3) and (DHA, $C_{22:6}$, *n*-3) are found mainly in fish (Kolanowski and Laufenberg, 2006; Russo, 2009).

The main sources of PUFAs are plant or seed oils, fish oil or marine oils and some microorganisms including fungi, yeast and bacteria. LNA is found in large amounts in flaxseed oil, it is also found in smaller amounts in soybean and canola oils. Most seed oils have high amounts of LA and have relatively little *n*-3 PUFAs, while EPA and DHA are found mainly in fish and also in varying amounts in other marine sources such as algae (Willis *et al*., 1998; Gunstone *et al*., 2007).

2.2.2.1. Edible Vegetable Oils

Since animal fat contain high amounts of saturated FAs, the consumption of which increases the risk for cardiovascular diseases (CVD), the consumption of vegetable oils was increased steadily over the past 25 years, while the demand for animal fat has declined (Cunnane, 2000; Russo, 2009). However, vegetable oils with unsaturated FAs are highly susceptible to oxidation, which could lead to off-flavor production as well as a decrease in their nutritional value. The vegetable oils currently produced in very large amounts in developed agricultural countries, contain variable amounts of PUFAs (Table 1), mainly LA, and also monounsaturated FAs, mainly oleic acid (Deckere *et al*., 1998). Canola oil contains only 10% LNA but its most abundant fatty acid is oleic acid (59%). Soybean oil contains high amounts of LA (52%) and also about 10% LNA. While Sunflower oil is highly unsaturated containing greater than 52% LA (Willis *et al*., 1998; Gunstone *et al*., 2007).

Table 1. Major fatty acid composition (w/w %) of selected vegetable oils.

Fatty acid	Canola[a]	Flaxseed (%)[c]	Soybean[b]	Sunflower[b]
$C_{16:0}$	5.0	5.7	10.0	6.0
$C_{18:0}$	2.0	3.3	4.0	6.0
$C_{18:1}$ *n*-9	59.0	23.7	25.0	33.0
$C_{18:2}$ *n*-6	21.0	14.4	52.0	52.0
$C_{18:3}$ *n*-3	10.0	52.6	7.0	< 1.0

[a]Adapted from Willis *et al*. (1998).
[b]Adapted from Gurr and Harwood (1991).
[c]Amount of fatty acid or lipid g/100 g sample.

Flax (*Linum usitatissimum)* is produced mainly for its fibre and oil. Flax is an economically important oilseed crop (Oomah, 2001; Lei *et al.*, 2003). The level of LNA in traditional flaxseed oil is generally higher than 50% of the total FAs (Daun *et al*., 2003; Bera *et al.,* 2006). Flaxseed oil also contains palmitic acid (<5%), stearic acid (5%), oleic acid (>18%) and LA (14%). Flaxseed oil containing essential fatty acids is cheap, plenty and highly used as edible oil in India, Asia and also in the Western world (Bera *et al.,* 2006). Flaxseed meal or oil can easily be

incorporated into common dietary items such as breads, rolls, cereals, muffins, margarines and salad dressings (Kew *et al.,* 2006).

Canada is considered the largest producer of flaxseed oil amounting to 40-50% of the total world production, while China, the United States and India account for another 40% of the world production (Oomah, 2001; Flax Council of Canada, 2009). The majority of flaxseed oil produced is used in chemical and pharmaceutical industries and for the production of paint. In Canada and most Western countries, flaxseed oil is consumed as a specialty item. However, 40% of the flaxseed oil produced in India is used for the human consumption (Bockisch, 1998; Bera *et al.,* 2006).

Flaxseed oil is rich in ω-3 PUFAs (Wallace *et al.,* 2003; Bozan and Temelli, 2008; Sharma *et al.,* 2009). Flaxseed oil contains LNA which is converted to the long-chain PUFAs, EPA and DHA that have proven to decrease the risk of some chronic diseases (Riediger *et al*., 2009). Dietary flaxseed oil is effective in preventing colon tumor development when compared with dietary corn oil containing mainly ω-6 fatty acids (Dwivedi *et al.,* 2005). Adequate intakes of ω-3 PUFAs are important for maintaining optimal tissue function in human and for meeting the demands of the fetus and neonate (Burdge and Calder, 2005). LNA is generally regarded as safe for public consumption in doses up to 3 g/day by the Food and Drug Administration (Harper *et al.,* 2006; Harris, 2007).

2.2.2.2. Fish Oil

Recent increase in the public's awareness and interest of the benefits of ω-3 PUFAs has lead the industry to expand and utilize marine oils, specifically fish oil, as a novel bio-ingredient and in the production of nutraceutical products (Shahidi and Wanasundara, 1998; Kolanowski and Laufenberg, 2006; Chakraborty *et al*., 2010). In the early years of the 20th century, fish oil was generally used in the leather industry, soap making and for the manufacture of paint. In the 1940's improvement in food processing technologies lead to the commercial productions of fish oil for human consumption. The consumption of fish oil increased in the 1980's through the evidence that ω-3 PUFAs have beneficial effects on CVD, based on an epidemiological study of the Greenland Eskimos (Haraldsson and Hjaltason, 2001; Leaf, 2008).

Fish is commonly classified into two groups based on the location of its oils namely, lean and fatty fish. Lean fish (e.g. cod) store their reserve lipids as TAGs in the liver, while fatty fish (e.g. herring, mackerel), store their TAGs in their flesh. Fish oil is produced as a by-product of the fishmeal industry. Fish lipids differ between species and according to diet, however; they are rich in long-chain polyunsaturated fatty acids (LC PUFAs) of the ω-3 family (Nadeau, 1992; Kolanowski and Laufenberg, 2006).

Fish is considered the main dietary source of LC PUFAs as well as fat soluble vitamins, such as vitamin A and D (Haraldsson and Hjaltason, 2001). Marine oils differ mainly from oils of plant and animal origin in that their fatty acid chain lengths have 20 to 22 carbon atoms, hence belonging to the ω-3 family, that consists of EPA ($C_{20:5}$ *n*-3) and DHA ($C_{22:6}$ *n*-3) (Russo, 2009).

Many studies showed that consumption of food products enriched with fish oil offers potential health benefits, especially in the protection against CVD (Jung *et al*., 2008), cancer (Ruxton *et al.,* 2005; Riediger *et al*., 2009) and improvement of brain development and function (Connor, 2000). However, there is a low acceptance of oily fish in many societies of the Western style diet, where the average fish intake is currently far below the recommended, minimum of two fish servings a week. Alternative ways to ensure an optimal ω-3 PUFAs intake is needed; one of the possibilities is the enrichment of food products with the various sources of ω-3 PUFAs, especially of marine origin (Kolanowski and Laufenberg, 2006). The United Kingdom Department of Health recommends intakes of 1.5 g of EPA and DHA per week (i.e. approximately 0.2 g per day), equivalent to two servings of fatty fish (Harris, 2007).

The main sources of EPA and DHA are fatty fish spp. such as herring, mackerel, sardine and salmon (Willis *et al*., 1998; Gunstone *et al*., 2007) their flesh usually contains a high proportion of fat tissues, while fish oil such as cod liver oil is marketed as a source of vitamins A and D, as well as EPA and DHA (Willis *et al*., 1998; Haraldsson and Hjaltason, 2001). The two most common sources of fish liver oil in the markets are cod liver and shark liver oils. The major FAs of some fish oils containing PUFAs are fully detailed below (Table 2).

Table 2. Major fatty acid composition (w/w %) of selected fish oils used in foods.

Fatty acid	Relative %[a] Atlantic herring[b]	Cod liver[b]	Mackerel[b]	Salmon[b]
$C_{14:0}$	8.8	2.8	7.8	2.9
$C_{16:0}$	14.8	11.6	16.1	10.7
$C_{18:0}$	1.0	25.2	1.8	3.6
$C_{18:1}$ *n*-9	16.5	2.5	12.9	24.5
$C_{18:2}$ *n*-6	0.8	0.7	1.3	5.2
$C_{18:3}$ *n*-3	0.4	2.2	1.1	5.3
$C_{20:4}$ *n*-6	< 1	< 1	< 1	7.6
$C_{20:5}$ *n*-3	< 1	9.3	7.6	4.5
$C_{22:6}$ *n*-3	< 1	8.6	7.7	17.0

[a]Amount of fatty acid or lipid g/100 g sample.
[b]Adapted from Willis *et al*. (1998).

2.2.2.3. Microbial Sources

Some fungal species such as *Mortierella* spp., including *M. elongate, M. isabellina, M. alpina* (Shimizu *et al*., 1989; Kendrick and Ratledge, 1992; Lounds *et al.,* 2007), are considered good source of PUFAs. Some *Phythium* and *Phytophtora* spp. also can produce ω-3 PUFAs (O'Brien *et al*., 1993). *Porphyridium cruentum,* the red marine algae produces significant amounts of EPA and AA (Willis *et al*., 1998; Ratledge, 2001; Sijtsma and Swaaf, 2004). However, only a small number of gram-negative marine bacteria such as *Flexibacter* and *Shewanella* sp. isolated from diverse sources including seawater, deep-sea sediments and the intestine of fish can produce PUFAs (Akimoto *et al*., 1990).

2.2.3. PUFAs Metabolism

LA and LNA the precursors of the ω-6 and ω-3 families, respectively, are required for growth, reproduction and health (Russo, 2009). They are considered essential fatty acids since animals cannot synthesize them, lacking the Δ^{12} and Δ^{15} desaturases and have to be obtained from the diet (Benatti *et al*., 2004; Russo, 2009). They can be elongated and desaturated to form the more unsaturated members AA and DHA. The *n*-3 and *n*-6 families are not interconvertible, but

compete for the same elongase and desaturase enzymes involved in their metabolism, and consequently the consumption of one family can affect the other (Russo, 2009).

The general metabolic pathways of *n*-3 and *n*-6 families in humans are shown in Figure 1. LNA is the preferred substrate for Δ^6 desaturase, however excess LA leads to a greater conversion of LA as compared to LNA (Burdge, 2004; Russo, 2009).

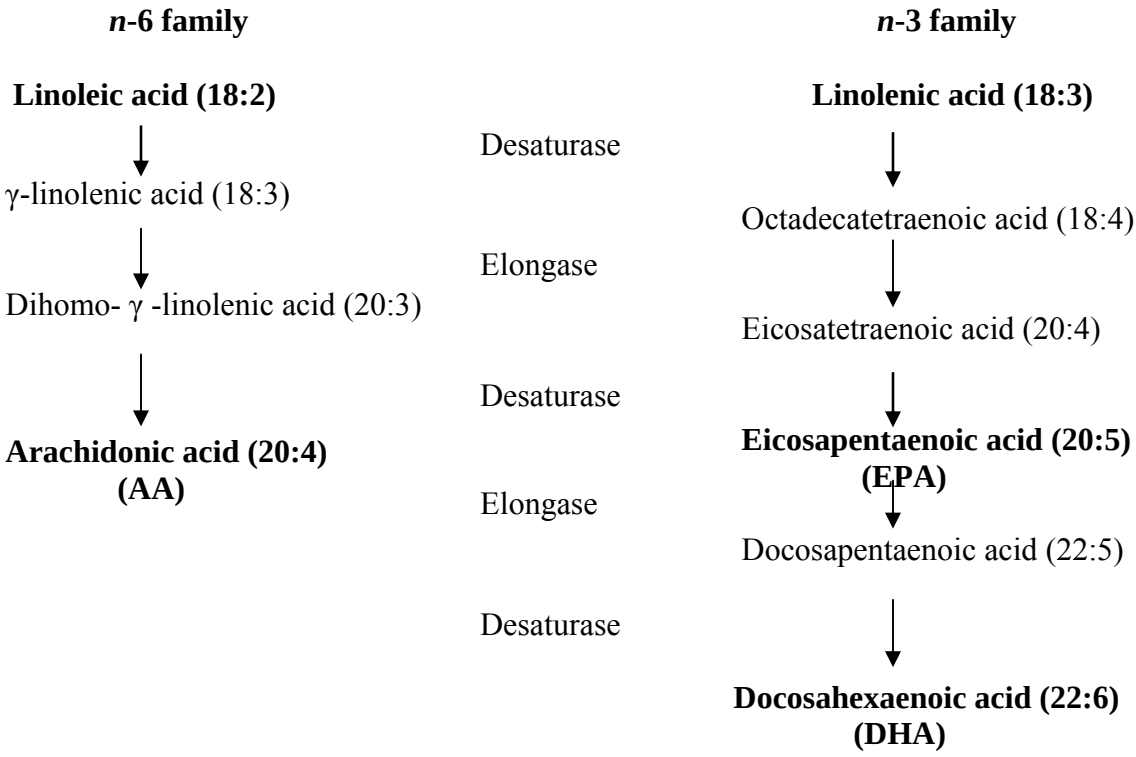


Figure 1. The metabolic pathway of PUFAs from ω-6 and ω-3 families of fatty acids.

2.2.4. PUFAs in Health and Diseases

PUFAs are incorporated into the cell membrane and contribute to the cell membrane fluidity, being highly unsaturated possessing low melting points; PUFAs decrease the membrane viscosity and affect several aspects of the membrane function (Russo, 2009). Hormone receptor binding to target enzymes such as ATPase activity is influenced by the membrane viscosity. PUFAs hydrolyze from the cell membrane phospholipids and are converted to eicosanoids, such as prostanoids and leukotrienes by cyclooxygenase and lipoxygenases, respectively (Russo, 2009). Eicosanoids are oxygenated derivatives of C_{20} FAs (AA and EPA) with potent hormonal

activities. Eicosanoids produced from AA are generally considered to be proinflammatory, while EPA produces eicosanoids that are generally anti-inflammatory (Larsson *et al*., 2004; Wong, 2005; Russo, 2009).

EPA and DHA can be converted into natural anti-inflammatory substances that help to decrease inflammation and pain (Harwood and Caterson, 2006; Russo, 2009; Zainal *et al*., 2009). Clinical studies have revealed that fish oil treatments relieve patients who are suffering from rheumatoid arthritis and inflammatory bowel disorders (Grimble, 2001; Riediger *et al*., 2009). In addition, preliminary research suggests that ω-3 PUFAs act against various human carcinomas that include breast, colon, skin, pancreatic, prostate, lung and larynx cancer via their ability to suppress eicosanoid production, enhancement of cell death and tumor cell growth inhibition (Rose and Connolly, 1999; Larsson *et al*., 2004; Roynette *et al*., 2004; Schley *et al*., 2007; Riediger *et al*., 2009). However, high intake of ω-6 PUFA can contribute to the development of colon and breast cancer (Larsson *et al*., 2004; Simopoulos, 2008).

CVD are the main cause of death and a major cause of premature death in the world (World Health Organization). ω-3 PUFAs lower the risk of CVD, probably by the multiple mechanisms of lowering serum TAGs, improving LDL:HDL ratio, anti-thrombotic effects and reduced endothelial activation (Bjerregaard *et al*., 2000; Baro *et al.,* 2003; Jung *et al*., 2008; Riediger *et al*., 2009). It was reported that fish oil intake decreases serum TAGs by inhibiting LDL and TAGs synthesis by the liver (Kris-Etherton *et al.,* 2002).

Fish oil ω-3 PUFAs are reported to associate with the brain development, also it is important for the vision and the functions of the reproductive system. This may be due to the fact that DHA is a component of brain nerve synapses, in the eye's retina, in the testes and sperms (Rice, 1996). It is mostly incorporated into the membrane phospholipids (McCann and Ames, 2005), hence it plays a vital role in the development and functions of these organs and systems. The nervous system contains approximately 35% PUFAs as its lipid content; most of which are LC PUFAs. In addition, higher prenatal intake of DHA has been shown to be associated with improved visual, cognitive, and motor development in off-spring (Jacobson *et al.,* 2008). Children given ω-3 PUFAs supplemented formula demonstrated enhanced visual and mental capabilities (Deckere *et al.,* 1998). While in human adults, clinical studies have suggested that low intake or inadequate

synthesis and metabolism of ω-3 PUFAs may be a common feature of disease processes such as schizophrenia, depression, Alzheimer's disease and memory loss (Ruxton *et al.*, 2005).

Despite the health benefits of ω-3 PUFAs, the mean daily intake falls far short of most recommendations, and a large percentage (up to 65%) of human population does not eat fish. Thus, there is a need for alternative sources of ω-3 PUFAs, such as functional foods and nutraceutical products (Kolanowski and Laufenberg, 2006; Shahidi, 2009). In addition, ω-3 PUFAs are highly unsaturated which makes them susceptible to oxidation, leading to off-flavors production, and resulting in the deterioration of the food quality. However, this can be overcome by the addition of a suitable antioxidant.

2.3. Phenolic Compounds

2.3.1. Sources and Structure

The term "phenolic" is chemically defined as a substance that contains an aromatic ring processing one or more hydroxyl substitute including functional derivatives (Rice-Evans *et al.,* 2003). Dietary phenolic compounds may be categorized into three main groups, phenolic acids including hydroxycinnamic acid derivatives and hydroxybenzoic acid derivatives, polyphenols (tannins) and flavonoids (King and Young, 1999). The first group hydroxycinnamic acid and its derivatives, mainly include *p*-couramic, sinapic, caffeic and ferulic acids (Harbone, 1998). An important hydroxycinnamic acid derivative is chlorogenic acid, an ester of caffeic acid, which is widely present in fruits and vegetables (Hollman, 2001). While hydroxybenzoic acid derivatives, include gallic and ellagic acids which are commonly found in berries and nuts. The second group, polyphenols are either polymers of catechin and epicatechin or polymers of gallic and ellagic acids. While the flavonoids group, include catechins, anthocyanidins, flavons and flavonols (King and Young, 1999). Catechins are widely found in tea leaves, while anthocyanidins are responsible for the bright colors of flower petals and fruits (Shahidi and Naczk, 1995b,c,d).

Fruit and vegetable intake is inversely correlated with risks of several chronic diseases in humans (Young *et al.,* 2005). Phytochemicals, and in particular phenolic compounds present in plant foods may be partly responsible for these health benefits through a variety of mechanisms, including antioxidant activity, antimutagenic and antibacterial properties (Young *et al.,* 2005).

Phenolic acids are present in many fruits and vegetables e.g. apple, black berry, raspberry, grapes, black currant, strawberry, onion and potato (Shahidi and Naczk, 1995d). Selected dietary phenolic acids are shown in Figure 2.

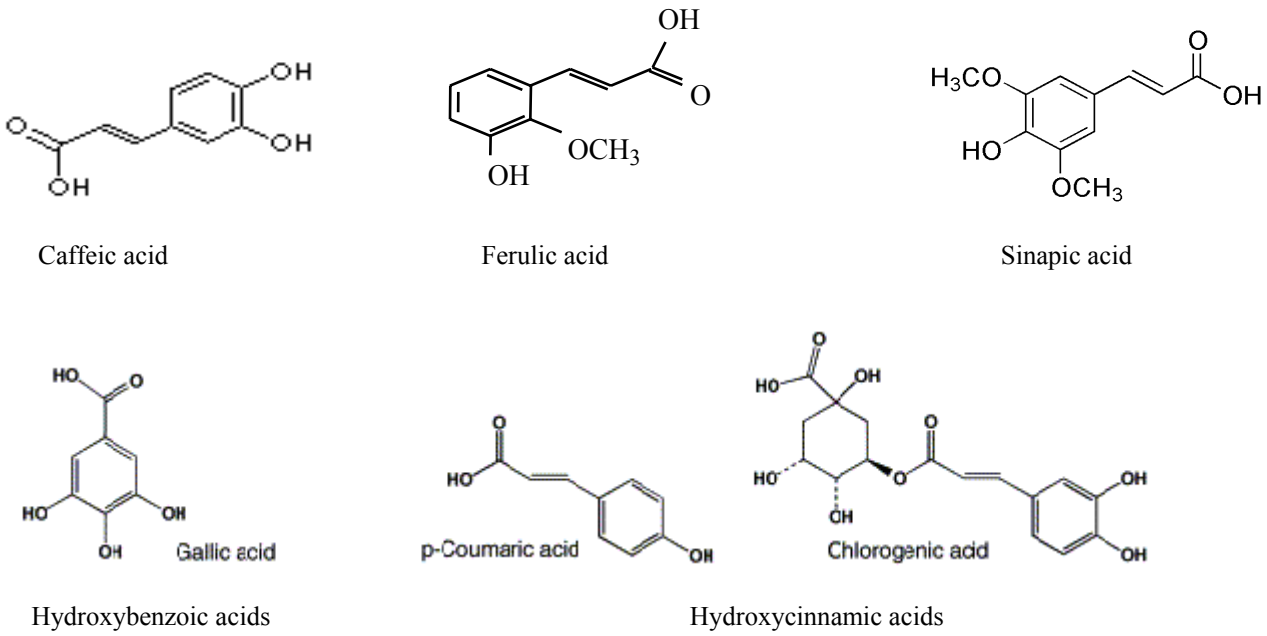


Figure 2. Structures of major phenols commonly found in food.

2.3.2. Nutritional and Antioxidant Properties

Antioxidants are compounds which when present in low concentrations in food or the body, delay or stop the oxidation of that substance (Shahidi and Naczk, 1995a). The most commonly used synthetic antioxidants are, Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), *t*-butylhydroquinone (TBHQ) and *n*-propyl gallate (PG), which have been widely used in the food industry (Howell and Saeed, 1999). These synthetic antioxidants are highly stable and inexpensive. However, due to their susceptibility of promoting carcinogenesis (Shahidi and Naczk, 1995a), research is focusing on the development and enhancement of natural antioxidants present in foods (Torres de Pinedo *et al.,* 2007). Most natural antioxidants, such as those found in plants, are phenolic in nature and possess varying antioxidant activity (Young *et al.,* 2005).

According to Pokorny (1991) natural antioxidants when compared to synthetic antioxidants, should have the following advantages: (i) they are readily acceptable by the consumers; (ii) they are considered to be safe; (iii) no safety tests are required by legislation; (iv) this natural

antioxidant is identical to the food which people have taken over a hundred years or have been mixing with food; and (v) this antioxidant not only stabilizes edible oils but also adds to the nutraceutical value of the oil.

Phenolic compounds are associated with nutritional and organoleptic qualities of foods from plant origin (Shahidi and Naczk, 1995b). Phenolic compounds at low concentration protect foods from autooxidation, but at high concentration, they can cause undesirable discoloration as a result of their interaction with the carbohydrate or protein components. This discoloration is called enzymatic browning as a result of phenols oxidation to quinones, which polymerize to form undesired colored pigments (Shahidi and Naczk, 1995c).

The autooxidation reaction of PUFAs is a three step reaction including initiation, propagation and termination (Fig. 3). The initiation step could be induced through the exposure of the lipid to light, heat, ionizing radiation, metal ions, and metalloprotein catalysts or by enzyme lipoxygenase (Cuppett *et al*., 1997).

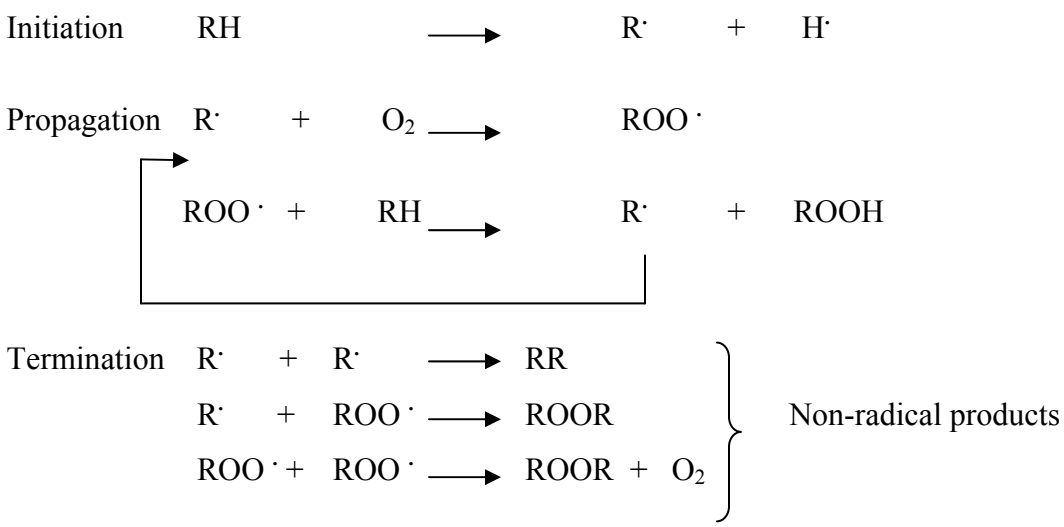


Figure 3. Autoxidation reaction, species identification is as follows: lipid (RH), Oxygen (O_2), free radicals, ($R^{\cdot}$ and $H^{\cdot}$), peroxy radical ($ROO^{\cdot}$) and non-radical products (RR, ROOR), including hydroperoxides (ROOH).

Phenolic antioxidants are able to hinder the oxidation process of the lipid by acting as free radical quenchers (Fig. 4).

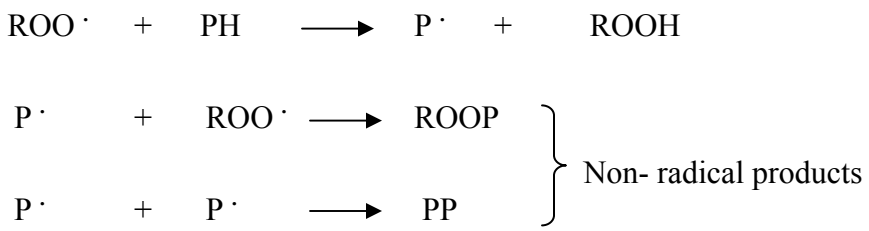


Figure 4. Radical scavenging action of phenolic antioxidants, where PH is the phenolic antioxidant, P · is phenolic radical, ROO · is the peroxide radical and ROOH is the hydroperoxide.

The phenoxy-radical intermediates formed are relatively stable due to resonance delocalization of unpaired electrons around the aromatic ring and the lack of appropriate sites for attack by molecular oxygen (Fig. 5).

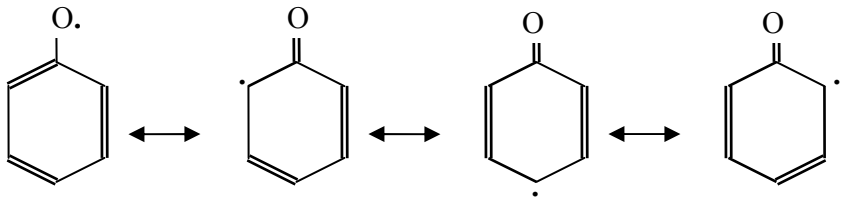


Figure 5. Phenoxy radical resonance stabilization.

Among naturally found phenolic compounds, phenolic acids are of high interest due to their potential biological properties (Torres de Pinedo *et al.,* 2007). Many phenolic acids are known to be potent antioxidants through their radical scavenging activity and due to their chemical structure, the reactivity of phenolic acids increases as the number of hydroxyl and methoxyl groups increases (Silva *et al.,* 2000). The consumption of fruits, vegetables and drinks such as tea and coffee, which contain phenolic compounds, has been linked to lower risk of some diseases; such as cancer and CVD (Yang and Landau, 2000; Luo *et al*., 2006; Kuriyama, 2008). However, the use of phenolic acids as natural antioxidants in foods and nutraceutical supplements has the limitation of low solubility in oily-based media (Stamatis *et al*., 2001). Nevertheless, lipase-catalyzed reactions of lipids with phenolic acids could produce structured lipids with phenolic moieties, which would have health benefits and improved solubility

characteristics (Akoh *et al.,* 1998; Stamatis *et al*., 2001; Sabally *et al*., 2007; Karboune *et al*., 2008; Karam *et al*., 2009).

2.4. Lipases

2.4.1. Definition

Lipases are an important group of biotechnologically relevant enzymes, and they find immense applications in food, dairy, detergent and pharmaceutical industries (Joseph *et al*., 2008). Lipases are defined as glycerol ester hydrolases that can hydrolyze tri-, di- and monoacylglycerols (Whitaker, 1996). They are water soluble but act on lipids, which are water insoluble, at the interface between oil and water (Lee and Akoh, 1996) and catalyze esterification, transesterification in addition to the hydrolytic activity on TAGs (Joseph *et al*., 2008).

2.4.2. Sources and Applications of Lipases

Lipases are present in animals, plants and microorganisms (Verger *et al*., 1990; Gupta and Roy, 2004). Animal lipases include pre-gastric esterase, pancreatic lipase and lingual lipases. Plants such as wheat germ and castor beans also contain lipases. Microbial sources include some yeasts (*Candida* and *Geotrichium*), moulds (*Rhizopus, Aspergillus)* and bacteria (*Bacillus, Pseudomonas)* (Hasan *et al*., 2006). Lipases are widely used because of their ready availability, low cost of production, utility in food, biotechnology and pharmacology (Verger *et al*., 1990; Song *et al*., 2006; Joseph *et al*., 2008). Novel biotechnological applications have been successfully established using lipases for the synthesis of biodiesel, the production of pharmaceuticals, agro-chemicals, and flavor compounds (Jaeger and Eggert, 2002). Moreover, the use of lipases in the food industry is increasing due to the need for the production of esters, biodegradable polyesters and specific FAs (Hasan *et al*., 2006; Zheng *et al*., 2009; Chakraborty *et al*., 2010).

In the last 15 years, lipase-mediated direct esterification and transesterification have motivated research on *Rhizomucor miehei* (Krmelj *et al.,* 1995), *Candida cylindraceae* (Gray *et al*., 1990) and *Candida antarctica* (Coulon *et al*., 1995) lipases. However, the literature indicated that the immobilized lipase from *Candida antarctica* appears to be suitable for the transesterification reactions in solvent-free medium (Sun *et al*., 2007; Zheng *et al*., 2009).

2.4.3. Lipase Structure and Catalyzed Reactions

The main structural features of lipases are α/ß-hydrolase fold consisting of a central, hydrophobic ß-sheet that is covered by α-helices from both sides, an active site formed by the Ser-His-Asp/Glu triad, and in most cases a "lid" formed by α-helix that covers the active site (Gandhi, 1997). Lipases undergo interfacial activation, which is attributed to the opening of a helical "lid" covering the active site when exposed to water-oil interface (Gandhi, 1997; Hou and Shimada, 2009) before catalyzing reactions. Lipase-catalyzed reactions can be classified into three groups which are hydrolysis, esterification and transesterification (Gandhi, 1997).

2.4.3.1. Hydrolysis

The hydrolysis of fats by lipases produces free FAs, monoacylglycerols, diacylglycerols and glycerol (Serri *et al.,* 2008). Lipase catalyzed hydrolysis can be used for the preparation of FAs from oils, especially for the selective hydrolysis and concentrations of PUFAs from edible oils (Willis *et al.,* 1998). The hydrolysis reaction takes place in the presence of water with the FAs on the glycerol backbone being replaced with hydroxyl group. Figure 6 shows a TAG hydrolysis process. Many authors reported the use of lipase-catalyzed hydrolysis reactions to produce concentrates of PUFAs from fish oils (Liu *et al.,* 2007; Chakraborty *et al*., 2010).

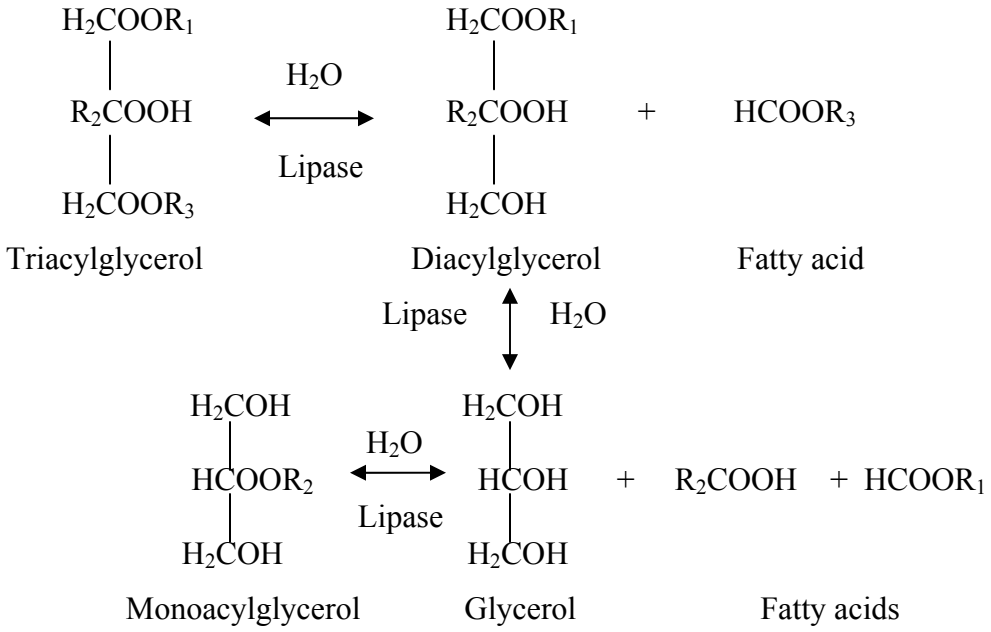


Figure 6. Enzymatic hydrolysis of triacylglycerols where R_1, R_2 and R_3 are different acyl groups.

2.4.3.2. Esterification

Esterification is generally described as a chemical reaction between an acid and an alcohol. It is the reverse reaction of hydrolysis and is used to synthesize selected products (Gandhi, 1997). The products of an esterification reaction are usually an ester and water (Fig. 7). Like other catalysts, enzymes affect rates of reversible reactions in both directions. The displacement of the equilibrium in reactions catalyzed by lipases is achieved by changing the concentration of the reactants, the main one being water. To favor synthesis, water should be kept as low as possible (Lortie, 1997). Direct enzymatic esterification of some primary alcohols and selected carboxylic acids were catalyzed by the *Candida antarctica* and *Rhizomucor miehei* lipases. The reactions were performed in solvent-free medium with the removal of water (Irimescu *et al*., 2004).

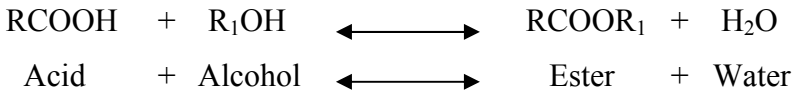


Figure 7. Esterification reaction.

2.4.3.3. Transesterification

Transesterification is a process of acyl exchange between two molecules. This process normally takes place between an ester and alcohol (alcoholysis), an ester and an acid (acidolysis) or an ester with another ester (interesterification), and no water is involved in the reaction, but some may be present in the reaction medium to maintain the lipase activity (Gunstone *et al*., 1994). Acidolysis is one of the most frequently used reactions to incorporate novel FAs into TAGs in several researches (Jennings and Akoh, 2001; Rocha-Uribe and Hernandez, 2004; Hamam and Shahidi, 2008; Hita *et al.,* 2009). While interesterification combines hydrolysis and esterification. The first step being the hydrolysis of the TAG molecule, followed by re-synthesis of the liberated FAs onto the glycerol molecule. Interesterification is another main strategy to incorporate PUFAs into TAGs. The literature reported extensive research work on the interesterification reaction (Gunstone, 1999; Kim *et al*., 2001; Akimoto *et al*., 2003; Osório *et al.,* 2009). The different transesterification reactions are as outlined below (Fig. 8).

(a) Alcoholysis

$R_1COOR_2 + R_3\text{-}OH \xleftrightarrow{\text{Lipase}} R_3COOR_2 + R_1\text{-}OH$

Ester Alcohol Ester Alcohol

(b) Acidolysis

$R_1COOR_2 + R_3COOH \xleftrightarrow{\text{Lipase}} R_1COOR_3 + R_2COOH$

Ester Acid Ester Acid

(c) Interesterifcation

$R_1COOR_2 + R_3COOR_4 \xleftrightarrow{\text{Lipase}} R_1COOR_3 + R_2COOR_4$

Ester Ester Ester Ester

Figure 8. Lipase-catalyzed transesterification reactions.

2.4.4. Specificity of Lipases

Specificity generally refers to an enzyme's ability to differentiate between several substrates competing for its active site. Lipases can be divided according to their specificity into three groups; (i) non-specific lipases, (ii) acyl group specific and (iii) positional specific lipases (Xu, 2000). Non-specific lipase can catalyze the release of FA from any position on the glycerol molecule. Acyl-group specific lipases catalyze the release of a particular type of FA from the TAG molecules, while positional lipases attack *sn*-1, 3 positions on the TAG molecule. The use of positional specific lipases has lead to the production of useful TAG mixtures whose composition could not be produced by simple chemical transesterification (Willis *et al*., 1998; Hamam and Shahidi, 2008). In recent years positional specific lipases have been intensively used in research purposes and food industry sectors (Kawashima *et al.*, 2001 and 2002; Zhou *et al*., 2001; Kim and Akoh, 2005 and 2006; Osório *et al.,* 2009*;* Zainal *et al*., 2009).

Lipase-catalyzed reactions can also be carried out at milder temperatures which is essential for heat sensitive FAs (Osório *et al.,* 2009). The food industry also widely employs the usage of lipases to improve the FAs composition of vegetable oils by incorporating EPA and DHA (Jennings and Akoh, 1999; Senanayake and Shahidi, 2004; Zainal *et al.,* 2009). Edible oils, such as flaxseed and fish oils are being used as a source of ω-3 PUFAs, and recent research carried out in our lab has focused on the enzymatic synthesis of structured phenolic lipids from these oils (Sabally *et al*., 2006b; Sabally *et al*., 2007; Karboune *et al*., 2008; Karam *et al*., 2009), which would have the numerous combined beneficial properties of both the PUFAs and phenolic acids.

2.5. Structured Lipids

2.5.1. Definition

Structured lipids (SLs) refer to the TAGs in which the composition and the distribution of their FAs at the glycerol backbone are modified (Akoh *et al.,* 1998; Osborn and Akoh, 2002). The modification of such FAs can occur through chemical or enzymatic means. However, the chemical methods are non-specific, require high temperature and usually result in randomly structured products. On the other hand, enzymatically generated SLs can result in products with FAs attached at specific positions (Osório *et al.,* 2009). The use of enzymes as ‘‘green’’ alternatives to produce high value-added SLs may offer significant improvements because of milder reaction conditions, higher selectivity and specificity, lower energy requirement, and purer products (Petersson *et al.,* 2005).

2.5.2. Synthesis and Properties

Lipid structural modification processes provide us with the ability to combine the beneficial characteristics of different FAs (Hita *et al.*, 2009). Structural changes can be designed to improve physical characteristics of fats for food formulation or for nutritive and therapeutic purposes targeting specific diseases and metabolic conditions (Osório *et al.,* 2009). Efforts have been focused on the production of SLs using medium chain fatty acids (MCFAs) and LC PUFAs, as well as the concentration of LC PUFAs from new and existing sources (Hita *et al.*, 2009). MCFAs are metabolized as quickly as glucose and produce twice the energy output of carbohydrates. SLs with MCFAs can be incorporated into formulated diets for surgery patients, the malnourished, infant formulae and as an energy supplement for athletes (Hita *et al.*, 2009). LC PUFAs, specifically DHA and AA, are faster absorbed in the human body when ingested in the form of SLs, thus there has been a great interest in producing SLs that contain such PUFAs (Hita *et al*., 2009; Osório *et al.,* 2009).

The nutritional quality of butter fat can be improved by changing hypercholesterolemic FAs such as lauric ($C_{12:0}$), myristic ($C_{14:0}$) and palmitic acids ($C_{16:0}$), with hypocholesterolemic fatty acid such as oleate ($C_{18:1}$) (Kermasha *et al*., 1995; Pabai *et al*., 1995). There are two commercially available SLs; Caprenin, which has similar fat properties to cocoa butter but with half the calories, Caprenin is composed of one molecule of behenic acid ($C_{22:0}$) and two molecules of either caprylic ($C_{8:0}$) or capric ($C_{10:0}$) acids. While Salatrim^{R} is a low calorie fat produced by the

interesterification of short-chain FAs with long-chain FAs developed by the Nabisco Food Group (Softly *et al*., 1994).

There has been great and numerous efforts at developing methods to concentrate PUFAs in marine oil and vegetable oils in OSM or in SFM (Osório *et al.,* 2009). Sridhar and Lakshminarayana (1992) were able to modify the composition of groundnut oil using 1,3 specific lipase from *Rhizomucor miehei*, decreasing the saturated long-chain FAs content by incorporation of EPA and DHA. In addition, Hita *et al.* (2009) reported that SLs with caprylic acid (CA) located at positions *sn*- 1, 3 and DHA at *sn*-2 were obtained, by the acidolysis of tuna oil and CA using *Rhizopus delemar* and *Rhizomucor miehei* lipases.

Nowadays, the edible oil industry depends widely on the rearrangement of lipids to obtain better melting and crystallization properties of oils, which make them suitable for their use in table spreads and shortenings. Additional uses of SLs are seen in food-grade emulsifiers, found as mono- and diacylglycerols (Krog, 1997; Fregolente *et al*., 2008).

2.6. Biocatalysis in Organic Solvent Media

Biocatalysis has become a useful alternative to chemical transformations for the production of a range of compounds with applications in food, chemical and pharmaceutical industries. However, it is not an easy task to obtain the desired levels of performance in terms of rate, yield and selectivity of the reaction (Gupta and Roy, 2004). Zacks and Kiblanov (1985) discovered that lipases can catalyze reactions in organic solvents with minimal water. However, the enzyme needs a thin monolayer of bound water to preserve its three dimensional structure which allows it to retain its catalytic activity in organic solvents (Zacks and Russell, 1988; Halling, 2002). Generally, hydrolysis reactions are favored in aqueous media, while in organic solvents synthesis reactions are predominant (Akoh *et al*., 1998).

Enzymes in organic solvents have manifested good selectivity and stability; however, catalytic activities in this environment are generally lower than in aqueous solutions (Gandhi *et al*., 2000; Schmid *et al*., 2001). This could be partly explained by the fact that in low water environments, enzymes are less flexible (Gomez-Puyou and Gomez-Puyou, 1998). This result in lower reaction rates in organic solvents compared to aqueous solutions (Klibanov, 1997).

On the other hand, the activities of enzymes also depend on the type of organic solvent, since some are known to inactivate or denature biocatalysts. With increasing concern for the environment, synthesis of SLs in solvent-free systems (Martinez *et al*., 2004; Rocha-Uribe and Hernandez, 2004; Sun *et al*., 2007; Chaibakhsh *et al*., 2009; Zheng *et al.,* 2009) and ionic liquids systems (Guo and Xu, 2005) have been extensively studied.

2.7. Biocatalysis in Solvent-Free Media

Enzymatic catalysis in solvent-free medium (SFM) has attracted considerable interest in recent years as an efficient approach to the synthesis of natural products, pharmaceuticals, fine chemicals and food ingredients. Under non-aqueous conditions, the industrial utility of enzymes can be improved, ease of product and enzyme recovery, and the ability to catalyze reactions that are unfavourable in aqueous solutions (Jin *et al.,* 2003). However, it would be technically beneficial if the enzymatic reactions were performed in mixtures of substrates in the absence of bulk solvents. Enzyme catalyzed reactions in SFM can combine the advantage of non-aqueous enzymology with high levels of productivity (Jin *et al.,* 2003).

The presence of free water in the reacting mixture may favour hydrolysis over synthesis, and some lipases do not exhibit any activity in water-saturated organic solvents (Torres and Castro, 2004). In addition, the utilization of organic solvents might limit the acceptability of nutraceuticals products and food ingredients. Therefore, there is a growing interest in studying lipase-catalyzed synthesis in SFM (Martinez *et al*., 2004). Although in a suitable solvent, the yield could be higher than that in aqueous media, solvents may generally inactivate the enzyme. Bosquet *et al.* (1999) synthesized unsaturated fatty acid glucoside esters for dermo-cosmetic applications in SFM, the method avoided the use of organic solvents and facilitated product purification.

Following the trend in applied biocatalysis towards solvent-free processes, lipases from various sources have been used for the transesterification of vegetable oils and alcohols (Dossat *et al*., 2002b; Köse *et al.,* 2002; Soumanou and Bornscheuer, 2003; Zeng*,* 2004). Lipase-catalyzed transesterification in SFM is important in industrial applications, and several studies reported that the immobilized *Candida antarctica* lipase (Novozym 435) could effectively catalyze the

transesterification of vegetable oils in SFM (Shimada *et al.,* 1999; Watanabe *et al.*, 2000; Weitkamp *et al*., 2006).

Enzymatic transesterification is a potential method for the modification of the physical and chemical properties of edible oils and fats (Dossat *et al*., 2002b). The transesterification of sunflower oil with butanol-1 by Lipozyme® was carried out in a SFM, and the reactor was maintained without any loss in activity for 3 months. This result was very different to that obtained using hexane, which leads to a total loss of the enzyme activity within a few hours. The mixture has interesting lubricant and surfactant properties (Dossat *et al.*, 2002b). In addition, the alcoholysis of cotton seed oil with primary and secondary alcohols was investigated using *Candida antarctica* lipase (Novozym 435) in a SFM, whereas under the optimized conditions a maximum methyl esters yield of 91.5% was achieved (Köse *et al*., 2002).

Monoacylglycerols (MAGs) and diacylglycerols (DAGs) are widely used as emulsifiers in foods, cosmetics, and pharmaceutical products (Fregolente *et al*., 2008). Often, mixtures of MAGs and DAGs are used in these applications because they are cheap and give proper performance. In addition, the use of DAGs replacing TAGs in food has been studied to prevent lipid accumulation in abdominal tissue, and therefore prevent some diseases related to obesity (Fregolente *et al*., 2008). Five lipases from *Thermomyces lanuginosus, Candida antarctica, Candida rugosa, Aspergillus niger,* and *Rhizomucor miehei* were screened to study their ability to produce MAGs and DAGs by the enzymatic glycerolysis of soybean oil in SFM. *Candida antarctica* lipase was the most efficient in producing DAGs and MAGs with maximum production of 48 and 32%, respectively (Fregolente *et al*., 2008).

Esterification of adipic acid with oleyl alcohol using *Candida antarctica* lipase was optimized in terms of time, temperature, agitation speed, and amount of enzyme using response surface methodology (RSM) (Chaibakhsh *et al*., 2009). Esters of adipic acid are a broad and diverse family of synthetic lubricant. Adipate esters are also used in other applications such as food packaging, plasticizers, perfumes, cosmetics, and coatings (Abdul Rahman *et al.,* 2008). In addition, adipate esters are highly biodegradable and thus more environmentally friendly (Gryglewicz, 2001). The ability to achieve a high percentage of yield (96%) and higher

volumetric productivity compared to OSM indicates that the SFM has a great potential for the enzymatic synthesis of adipate ester (Chaibakhsh *et al*., 2009).

SLs have been receiving increasing attention in the food area, since they may be a good vehicle for providing nutraceutical FAs to consumers. Jennings and Akoh (2001) reported that the esterification of menhaden fish oil with capric acid using *Rhizomucor miehei* lipase resulted in a 36.7% bioconversion yield in SFM. Further studies on the enzymatic synthesis of structured phenolic lipids in SFM have also been conducted by Guyot *et al*. (1997). Phenolic acids from green coffee have useful natural antioxidant properties, its esterification with fatty alcohols resulted in the formation of more lipophilic constituents that can be used as a nutraceutical product (Guyot *et al*., 1997). In addition, feruloylated mono- and diacylglycerols were synthesized in SFM using *Candida antarctica* lipase, and the yield was 96% (Sun *et al*., 2007). Zheng *et al*. (2009) reported the optimization of phenolic lipids synthesis from ethyl ferulate and triolein in SFM using *Candida antarctica* lipase, and the highest bioconversion yield was 73.9%.

Lipase-catalyzed synthesis in SFM has a number of advantages as compared to that in OSM, including the use of a smaller reaction volume, maximization of substrate concentration and with no additional solvent-recovery. In addition, downstream processing is easier as fewer purification steps are required providing significant cost savings, as well as toxic organic solvents are completely avoided (clean conversions), and an increase in the volumetric productivity can be achieved (Dossat *et al.,* 2002a; Fregolente *et al*., 2008). Lipases have been recently used in SFM, either in direct esterifications or in transesterifications, for the synthesis of esters, sugar esters, phospholipids or glycerides (Guyot *et al.,* 1997; Weber and Mukherjee, 2004; Sun *et al*., 2007; Fregolente *et al*., 2008; Chaibakhsh *et al*., 2009; Xin *et al.,* 2009). However, there are some problems with the use of SFM, mainly, the high viscosity of the medium as well as the production of high amounts of glycerol, free FAs as by-products. These by-products affect the reaction equilibrium and limit the mass transfer rate (Dossat *et al.,* 1999; Zheng *et al.*, 2009). Thus the development of a bioprocess for the lipase-catalyzed synthesis in SFM is of major interest, but with great challenge.

2.8. Enzymatic Synthesis of Phenolic Lipids

Phenolic lipids are SLs that possess a polyunsaturated hydrocarbon chain and an aromatic ring bearing one or more hydroxyl or methoxyl substitutes, attached via an ester bond (Lue *et al.,* 2005). These novel structured molecules could offer the numerous combined beneficial properties of both PUFAs and phenolic acids, including decrease the risk of cardiovascular, autoimmune, inflammatory diseases and some cancers (Calder, 2001; Larsson *et al*., 2004; Riediger *et al*., 2009) with enhanced anti-oxidative, improved solubility and functional properties (Stamatis *et al*., 2001; Sabally *et al*., 2007).

Aromatic esters of hydroxycinnamic acid derivatives, such as phenylethyl- or tyrosyl-ferulate, are found in natural sources such as bee propolis (Ahn *et al*., 2004) and plants (Yang *et al*., 2003), they have been found to have antioxidant, anticancer and have anti-fungal/microbial activities (Stevenson *et al*., 2007). Phenolic antioxidants have been broadly employed for food preservation (Torres de Pinedo *et al.,* 2007). At the same time, some of these phenols, present in natural sources, have revealed interesting biological properties, anticancer and heart-protecting as well as exhibits anti-inflammatory properties (Torres de Pinedo *et al.,* 2007). However, all naturally occurring antioxidants are strongly hydrophilic and this makes their incorporation into fat and oil matrices difficult. This problem is being approached by the preparation of lipophilic antioxidants from these natural sources, e.g. isoflavone fatty acid esters (Lewis *et al.,* 2000), hydroxytyrosol fatty acid esters (Trujillo *et al.*, 2006). These antioxidants were prepared enzymatically using *Candida antarctica* lipase (Torres de Pinedo *et al.,* 2007).

Lipase-catalyzed synthesis of phenolic lipids can be classified as 'natural' and the reactions can be carried out under mild conditions which can be applied in food industry (Humeau *et al*., 1995). The enzymatic synthesis of phenolic lipids has been reported in the literature (Guyot *et al.,* 1997; Stamatis *et al.,* 1999; Compton *et al*., 2000; Lue *et al.,* 2005; Karboune *et al.,* 2005; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al*., 2009). The esterification of cinnamic or *p*-hydroxyphenyl acetic acid with short or MCFAs in SFM using *Candida antarctica* lipase, resulted in high yields of 82 and 97%, respectively, after 12 days (Stamatis *et al*., 1999). In addition, Compton *et al*. (2000) reported that the transesterification of ethyl ferulate with triolein using Novozym 435 produced 44% combined yield of feruyl monoolein and feruyl

diolein, while using three-fold excess of triolein, a 77% combined yield of feruyl monoolein and feruyl diolein was achieved.

Most phenolic acids, such as ferulic, caffeic and other cinnamic acid derivatives, are known to be potent antioxidants, possibly through their ability to scavenge free radicals (Silva *et al*., 2000). The potential use of phenolic acids as nutraceuticals in lipid based media and supplements are, however, limited because of their low solubility in these conditions (Stamatis *et al*., 1999). The formation of esters or structured lipids from lipids and phenolic acids could result in the formation of novel products with improved solubility, stability and potential health benefits of both the lipid and the phenolic acid.

Generally, the esterification of the cinnamic acid derivatives and benzoic acid derivatives is affected by electron donating substituents on the aromatic ring (Buisman *et al*., 1998). The electron donating ability of hydroxyl and methoxyl groups were reported to interfere with the nucleophilic attack of the carboxylic acid of the phenolic compound, as a result, very low bioconversion yields were obtained, when caffeic (3,4-dihydroxycinnamic acid) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) was esterified with butanol. While 97% bioconversion yield was obtained with cinnamic acid (Guyot *et al*., 1997; Stamatis *et al*., 1999). The steric hindrance caused by the large substituent group of methoxyl can also affect the reaction. However, when the side chain on the aromatic ring was saturated (*p*-hydroxyphenyl acetic acid), *p*-hydroxylation had less effect on lipase activity (Guyot *et al*., 1997; Stamatis *et al*., 1999).

Research carried out in our laboratory has succeeded in the enzymatic synthesis of phenolic lipids, using Novozym 435 in OSM, including lipase-catalyzed esterification of fatty alcohols and phenolic acids (Lue *et al*., 2005) where a 100% bioconversion yield was obtained from the esterification of cinnamic acid and oleyl alcohol. Karboune *et al.* (2005) reported the transesterification of cinnamic acid with triolein, and a combined yield (42%) was obtained of monoley1-3-cinnamate and dioleyl-2-cinnamate. The enzymatic esterification of dihydrocaffeic acid with linoleyl alcohol was investigated, and a maximum esterification yield of 99.3% was obtained (Sabally *et al.,* 2005). Also the transesterification of selected phenolic acids with edible oils, including flaxseed and fish liver oils by Novozym 435 (Sabally *et al.,* 2006b and 2007; Karboune *et al.,* 2008) resulted in the production of both phenolic mono- and diacylglycerols in a

solvent mixture of hexane:2-butanone with 75:25(v/v), and the synthesized phenolic lipids demonstrated radical scavenging activity compared to that of α-tocopherol.

In addition, Karam *et al.* (2009) reported that lipase-catalyzed acidolysis of fish liver oil with 3,4-dihydroxyphenyl acetic acid using Novozym 435 was investigated. A maximum bioconversion yield of 83% was obtained, in a solvent mixture of hexane:2-butanone with 75:25 (v/v). The results showed that the acidolysis reaction resulted in an increase of $C_{20:5}$ ω-3 and $C_{22:6}$ ω-3 proportions from 11.5 and 20.2% in the original fish liver oil to 22.6-27.1 and 22.8-23.1% in the synthesized phenolic lipids, respectively.

2.8.1. Parameters Affecting the Enzyme Activity and Product Yield

2.8.1.1. Effect of Water Activity

The variation in the activity of a biocatalyst can be correlated with the thermodynamic water activity (a_w). This can be measured via the vapour phase above the reaction mixture, and can be expressed as the partial pressure of the solution over the partial pressure of pure water measured through the vapour phase (Valivety *et al*., 1992). At equilibrium, a_w values for all the phases in a system are equal, regardless of the critical water content of individual systems.

Water is required for enzyme action; it participates directly or indirectly in all non-covalent interactions maintaining the native, catalytically active enzyme conformation (Zacks and Klibanov, 1985). Generally, the rate of reaction is very low or zero in dry conditions and increases with controlled addition of water reaching maximum activity, followed by a decline in reaction rate (Valivety *et al*., 1992).

The enzymatic activity strongly depends on the amount of water absorbed by the enzyme (Zacks and Russel, 1988) as this is linked to the thermodynamical a_w (Valivety *et al.,* 1992). At low water activity an increasing amount of water favours the optimal conditions of the enzyme, whereas high water activities have a negative influence on the position of the reaction equilibrium and on the aggregation behaviour of the enzyme, causing limited mass transfer (Valivety *et al.,* 1992). To characterize the relationship between water and the other components in the micro-aqueous reaction system, research has been done on the effect of a_w on enzyme

activities and bioconversion yields (Schmid *et al*., 1999; Secundo and Carrea, 2003; Guo and Xu, 2005; Lue *et al*., 2005).

Karra-Chaabouni *et al.* (2002) have investigated the relation between the enzymatic activity and water activity for the synthesis of geranyl butyrate by *Rhizomucor miehei* esterase in SFM. The bioconversion yield was about 75% for different low initial a_w of 0.2 to 0.5 but decreased to 13% for a_w above 0.5. This behaviour was related to water-enzyme interactions. In addition, lipase-catalyzed transesterification of ethyl ferulate with triolein to produce ferulyl oleins in SFM was investigated (Xin *et al.,* 2009), and the water activity had an obvious influence on the transesterification yield. A maximal combined yield of 29.0% of ferulyl diolein and ferulyl monoolein was obtained at 60°C and a_w of 0.75.

2.8.1.2. Effect of Reaction Temperature

Temperature can affect the enzyme activity where an increase in the temperature leads to an increase in the bioconversion yield; however higher temperatures may denature the lipase protein structure (Stamatis *et al.,* 1999 and 2001; Biselli *et al.,* 2002). Arcos *et al*. (1998) investigated the enzymatic synthesis of acylglycerols from LNA and glycerol by Novozym 435 in SFM. The bioconversion yield was increased with the increase in temperature in the range of 30 to 70ºC. However, deactivation of the enzyme occurred when the temperature was set at 70ºC. Previous research has shown that the optimal temperature of Novozym 435 activity for the enzymatic synthesis of phenolic lipids was in the range of 45 to 60ºC (Guyot *et al.,* 1997; Buisman *et al.,* 1998). Also Lue *et al*. (2005) reported that the highest initial enzyme activity of Novozym 435 was observed at 55°C for the esterification of cinnamic acid and oleyl alcohol in OSM.

2.8.1.3. Effect of Enzyme Concentration and Agitation Speed

Normally, as enzyme concentration increases, the reaction equilibrium will be shifted towards the synthesis direction quickly. After reaching certain enzyme concentration, the bioconversion yield is constant. Awang *et al.* (2004) reported that an excess of Novozym 435 (> 0.2 g) did not increase the esterification yield of oleic acid with oleyl alcohol. In addition, Carrín and Crapiste (2008) reported that in Lipozyme IM-catalyzed acidolysis of sunflower oil with palmetic acid (PA) and stearic acid (SA) mixture, the extents of PA and SA incorporations were enhanced by

increasing the amount of enzyme in the reaction, but a significant increase was not observed when the enzyme was greater than 8% by weigh of substrates.

The agitation speed may decrease the boundary liquid layer surrounding the porous support, resulting in lower diffusional limitations (Almeida *et al*., 1998; Barros *et al*., 1998). Hadzir *et al*. (2001) reported that an agitation speed of 150 rpm was selected for the enzymatic alcoholysis of triolein by Novozym 435. A similar result was reported by Lue *et al.* (2005) for the esterification of cinnamic acid and oleyl alcohol in OSM, using the same enzyme.

2.8.1.4. Addition of Silica Gel

Silica gel is an amorphous form of silicon dioxide, by definition it functions as a dessicator. No chemical reactions of side products take place making the use of Silica gel appealing (Castillo *et al.,* 1997; Halling, 2002). Among various studies on the enzymatic alcoholysis of vegetable oils, the main difficulty that has been reported is the insolubility of free glycerol in oil or organic solvent, which can inhibit the reaction by limiting the substrate and product diffusion. To solve this problem Silica gel has been used, which allow glycerol extraction from the reaction mixture (Stevenson *et al*., 1994). In general, Silica gel is used as an adsorbent for glycerol during the enzymatic reactions, and it is now well documented that Silica gel addition would result in an enhancement in lipase-catalyzed esterification reactions (Halling, 2002).

Karboune *et al.* (2005) investigated the addition of Silica gel as an adsorbent for glycerol in the bioconversion of cinnamoylated lipids. An increase in the maximum bioconversion yield by 17% was obtained with the addition of 2.2 mg of Silica gel/mL to the reaction mixture. However, increasing the Silica gel concentration to 4.4 mg/mL resulted in a 26% decrease in the bioconversion yield, and this decrease may be due to the dehydration of the enzyme by the Silica which led to an enzyme deactivation (Stevenson *et al*., 1994).

2.8.1.5. Addition of Surfactants

Surfactants decrease the surface tension and hence they increase the interfacial area between substrates and enzyme molecules; these correlations would presumably harbor more lipase molecules, where the increase in the solubility of substrates would consequently increase the rate of the enzymatic reaction (Goto *et al.,* 1994; Liu *et al*., 2000). Several strategies have been

employed to enhance the enzymatic activity. One way to increase the enzyme activity is to create a homogeneous system by the solubilization of the enzyme in the organic solvent (Larsson *et al.,* 1990). Different surfactants, such as dialkyl glucosyl glutamates, Span 60 has been used to create surfactant-coated lipases, which when suspended in organic solvents have been reported to show increased enzyme activity due to increased solubility of the enzyme (Okahata and Ijiro*,* 1988).

Surfactant-coated enzymes have been extensively studied as promising biocatalysts for synthesis reactions in anhydrous organic solvents (Kamiya *et al.,* 1995; Gamez-Meza *et al*., 2003). Enhancement of lipases depends on the type of surfactants where a strong interaction between the cationic head group in the surfactant molecule and the negatively charged lipase might induce a change in the three dimensional structure of lipase, which may result in a reduced activity (Kamiya *et al.,* 1995; Xia *et al.,* 1996; Liu *et al*., 2000). On the other hand, higher enzymatic activity was obtained with the non-ionic surfactant coated lipase than that with anionic or cationic surfactants (Liu *et al*., 2000). Interaction between the non-ionic surfactant and the lipase via a weak interaction, would favor the maintenance of the stereoconfiguration of lipase for better catalytic performance, which would result in a higher bioconversion yield (Goto *et al.,* 1994). The surfactant-coated lipases can effectively catalyze many synthesis reactions in OSM (Goto *et al.,* 1994; Kamiya *et al*., 1995).

Transesterification between MCFA and long-chain FAs triacylglycerols in SFM was investigated, using surfactant-coated lipase which was a complex of (*Rhizopus japonicus* lipase and Span 60), a 74% bioconversion yield was obtained after a 48 h reaction period (Mogi *et al.*, 2000). In addition, Thakar and Madamwar (2005) reported that *Candida rugosa* lipase was coated with different surfactants and immobilized on Silica to catalyze esterification reaction for ethyl butyrate production, a flavor ester. Among the surfactants tested the non-ionic surfactant (Span 65) performed better than the cationic surfactant (CTAB), whereas an inhibitory effect was observed with the anionic surfactant (AOT). In another study, a surfactant-coated lipase was prepared by mixing *Candida rugosa* lipase with Span 60; it was used to hydrolyze tuna oil efficiently in a two-phase aqueous-organic system (Ko *et al.,* 2006).

2.9. Response Surface Methodology

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes, and optimization of multiple variables to predict the best performance conditions with minimum number of experiments (Mutalik *et al.,* 2008). The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results, so it is considered a faster and less expensive method for gathering research data than the classical method (Gunawan *et al*., 2005; Rodrigues *et al.,* 2008). It is also a useful statistical technique for designing experiments and analyzing the effects of independent variables (Garrido-Vidal *et al.,* 2003; Nemukula *et al.,* 2009). Recently, optimization of lipase-catalyzed production of various esters by RSM has been investigated (Macedo *et al*., 2004; Keng *et al*., 2005; Sharma *et al.,* 2009; Zheng *et al.,* 2009). In addition, this method is efficient to explain the relationships between different variables and the response when there is interaction between the variables (Gunawan *et al*., 2005; Abdul Rahman *et al.,* 2008).

RSM consists of an empirical modelization technique, which has been used to evaluate the relation between experimental and predicted results (Chowdary *et al*., 2002). Central composite rotatable design (CCRD) is generally considered as the most appropriate design for response surface optimization (Jeong and Park, 2006).

Groundnut oil is one of the major edible oils, but it does not contain ω-3 PUFAs, while flaxseed oil is a good source of ω-3 PUFAs, but it is not generally used for cooking purposes because of its high PUFAs content. Groundnut oil can be enriched with ω-3 PUFAs extracted from flaxseed oil by lipase-catalyzed acidolysis (Sharma *et al.,* 2009). SLs containing ω-3 PUFAs was produced by incorporating LNA from flaxseed oil into groundnut oil using Lipozyme IM from *Rhizomucor miehei* in hexane. The reaction conditions were optimized by RSM using a four-factor five-level CCRD. Optimal conditions for the reaction were determined to be; enzyme concentration 3.75% (w/w), temperature 37.5°C, incubation time 30.8 h, and the ratio of LNA to groundnut oil was 1:16 (w/w). In addition, Zheng *et al*. (2009) reported the optimization of phenolic lipids synthesis in SFM using RSM, where feruloylated diacylglycerol was synthesized using Novozym 435 by the transesterification of ethyl ferulate and triolein. RSM was applied to optimize the reaction, and a total of 32 experiments were performed to study the reaction

temperature, time, enzyme load, water activity and substrate molar ratio. The highest bioconversion yield was 73.9%, at 55°C, 5.3 day, enzyme load of 30.4 mg/mL, water activity of 0.08, and a substrate molar ratio of 3.7.

2.10. Characterization of Phenolic Lipids

2.10.1. Generality

It has been literatured that various analytical techniques, including thin-layer chromatography (TLC; Buisman *et al.,* 1998; Lee *et al.,* 2004; Sabally *et al.,* 2007), high-performance liquid chromatography (HPLC; Guyot *et al.,* 1997; Stamatis *et al.,* 1999; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009), gas-liquid chromatography (GLC; Buisman *et al.,* 1998) and liquid chromatography mass spectrometry (LC-MS; Compton *et al.,* 2000; Christophoridou *et al.,* 2005; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009) have been used widely for the structural analysis of phenolic lipids. In addition, various techniques can be used to determine the antioxidant properties of phenolic lipids (Chen *et al*., 1992; Silva *et al*., 2000; Sabally *et al.,* 2007; Karam *et al*., 2009).

2.10.2. Thin-Layer Chromatography

Thin-layer chromatography (TLC) separation has been used for the qualitative analysis of phenolic lipids products and for preparative separation of products, which can be further analyzed by HPLC or GLC methods. Separation is generally carried out on a glass plate with a layer of adsorbent, usually Silica gel. Separated fractions can be visualized by eye if the spots are colored or by spraying with 20% H_2S0_4. Alternatively, plates with fluorescence incorporated into their stationary phase can be visualized under UV after developing (Stamatis *et al.,* 1999; Sabally *et al*., 2007; Karam *et al*., 2009). Silica gel 60 plates were used for the qualitative analysis of reaction mixture from the esterification of phenolic acids with alcohols (Stamatis *et al.*, 2001). Elution of the sample fractions was carried out using a solvent mixture of chloroform/methanol/acetic acid mixture (63:2:1, v/v/v) and visualized by spraying the plates with 5% ethanolic solution of H_2S0_4, then heating for 10 min at 150°C or visualized under UV lamp. In addition, Karam *et al.* (2009) reported that synthesized phenolic lipids from fish liver oil and 3,4- dihydroxyphenyl acetic acid were separated using TLC, with a saturated solvent mixture of hexane/diethyl ether/acetic acid (70:30:1, v/v/v), visualized by spraying the plates with 20%

of H_2S0_4, and heating for 10 min at 150°C or visualized under UV lamp as indicated previously by Lee *et al.* (2004).

2.10.3. High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) has often been used over other instrumentation, and has shown scientifically to be the overall preferred method of choice for quantification and separation of phenolic lipids (Karboune *et al.,* 2005; Sabally *et al.,* 2006 a,b and 2007; Karam *et al*., 2009). Generally reversed phase HPLC has been used for separation of phenolic lipids and it uses a predominantly polar solvent to elute samples from a mainly non-polar stationary phase (Sabally *et al*., 2007; Karam *et al*., 2009). The most widely used stationary phase for this purpose is the octadecylsilylsilica (ODS) or as it is commonly called C_{18}. It is prepared by the reaction of the surface silanol groups of Silica with an organochlorosilane (Pomeranz and Meloan, 1994). The main interactions between the sample components and the stationary phase are dispersion forces. Non-polar molecules are retained by the stationary absorbent more than polar molecules (Sewell, 1992); hence, C_{18} columns should separate polar phenolic acids from mainly non-polar lipids and phenolic lipids products.

Phenolic lipids products were separated using an elution solvent mixture of methanol/water from a C_{18} Nucleosil column (Stamatis *et al*., 1999 and 2001) with 280 nm detection. UV detection at 280 nm was combined with evaporative light scattering detector for the determination of phenolic lipids products eluted using an isocratic solvent gradient of methanol/water from a C_{18} column (Karboune *et al*., 2005; Lue *et al*., 2005). Sabally (2006b) reported that the transesterification reaction of flaxseed oil and dihydrocaffeic acid using Novozym 435 was monitored by HPLC analysis, according to the modified method of Andrikopoulos *et al.* (1991). The separation of reaction components was performed on a Zorbax SB-C_{18} reversed-phase column and ultraviolet diode-array (UV-DAD) detector at 280 and 215 nm, and the elution was carried out by a gradient system using acetonitrile/methanol mixture (7:5, v/v) as solvent (A) and isopropanol as solvent (B). Using similar elution conditions, samples were further structurally characterized by HPLC-atmospheric pressure chemical ionization mass spectroscopy (APCI-MS).

Recent research on phenolic lipids has used APCI-MS as well as electrospray ionization (ESI) techniques; in combination with liquid chromatography-mass spectrometry (LC-MS) (Compton *et al.,* 2000; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). LC-MS is considered to be one of the most powerful techniques used for the characterization of biomolecules due to its high sensitivity and specificity. Generally its application is oriented towards the specific detection and potential identification of chemicals in a complex mixture. LC-MS has been used for the structural characterization of lipids and phenolic lipids (Andrikopoulos, 2002; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009).

2.10.4. Gas-Liquid Chromatography

Gas liquid chromatography (GLC) is used to separate compounds, which can be volatilized directly or via derivation. GLC uses a mobile gas phase to pass sample through a column mostly a capillary that contain a thin film of stationary liquid phase (Jennings, 1984). The most commonly used stationary phases are the polysiloxanes, especially the apolar dimethyl polysiloxanes. Separations are generally based on boiling points, which are governed by chain length and the level of unsaturation. Samples are methylated prior to injection to increase their volatility and stability during the GLC analysis. Injected samples dissolve in the liquid stationary phase are carried through the column by a carrier gas. Compounds that are more strongly attracted to the stationary phase take a longer time to emerge from the column than those that are less attracted to the column (Jennings, 1984). Detection of compounds can be carried out using flame ionization detection (FID) or by mass spectroscopy (MS).

GLC analysis has been conducted for the quantification of lipase-catalyzed SLs for the determination of their FAs composition (Willis *et al.,* 1998). Components of a reaction mixtures containing phenolic lipids were analyzed by GLC on Supelco Omega Wax fused capillary column linked to an FID detector (Sabally *et al.,* 2006b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009).

2.10.5. Antioxidant Properties Determination

Oxidative stress, is defined as the imbalance between oxidants and antioxidants in favor of the oxidants (Sies, 1991), which has been suggested to be the cause of aging and various diseases in

humans (Halliwell, 2007). Hence, the balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system (Tiwari, 2001). Recently, research associated with natural antioxidants has increased dramatically in various fields, including food chemistry, food biology, medicinal plants, and biochemistry (Moon and Shibamoto, 2009). As a result, the measurement of the antioxidant activity has become a focal point by both the food industry and by health professionals (Shahidi and Naczk, 1995a,b,c;d; Shahidi and Wanasundara, 1998), and there is a large literature on the activity of natural antioxidants and stability evaluations of different unsaturated food lipids (Moon and Shibamoto, 2009).

Edible oils contain unsaturated FAs, which are extremely susceptible to autoxidation, affecting the quality and shelf life of such oils (Läubli and Bruttel, 1986). Lipid oxidation is a serious problem in food because it produces rancid odors and flavors, decreases shelf life, alters colour and decreases the nutritional value (Addis and Warner, 1991). The ability of a compound to inhibit lipid oxidation in foods is thought to be related to its free radical scavenging activity, and the protection of lipid oxidation is a critical factor to food quality and shelf-life of edible oils (Alamed *et al*., 2009). Most antioxidant tests associated with lipid peroxidation involve the monitoring of hydroperoxides or a specific oxidative secondary product. There have also been many reviews that summarize various antioxidant assays involved in the investigation of lipid peroxidation (Rael *et al*., 2004; Yoo *et al.,* 2007; Moon and Shibamoto, 2009).

The oxidation of food lipids can be measured at different stages, including the initiation stage, where there is a loss of unsaturation as measured by a decrease in the Iodine Value. The propagation step can be followed by measurement of the accumulation of primary peroxidation products via the determination of the peroxide value (PV) (AOCS, 1989), which gives an indication of the oxidative status (Gotoh *et al.,* 2006). The termination stage can be studied by measurement of general secondary carbonyl and hydrocarbon compounds, or presence of specific types of products such as aldehydes (anisidine value; *p*-AnV) and malonaldehyde (thiobarbituric acid reactive substance; TBARS) (Handelman and Pryor, 1999; Yoo *et al.,* 2007).

The antioxidant properties of a compound can be measured either directly using the free radical method (Dudonné *et al.,* 2009) or indirectly using TBARS and *p*-AnV, which measure secondary

oxidative products (Handelman and Pryor, 1999). The free radical assay using 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) is an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts (Sanchez-Moreno, 2002). Most phenolic compounds have antioxidant properties through their ability to scavenge free radicals. Generally they have hydrogen atoms they can donate and the resultant phenoxy radical is stabilized by resonance (Rice-Evans *et al*., 1996).

The antioxidant activity of potato peel extract using the free radical method ($DPPH^{\bullet}$) was found to be comparable to BHT (Kanatt *et al*., 2005). In addition, Silva *et al.* (2000) reported the scavenging activity of caffeic and dihydrocaffeic acid towards the free radical $DPPH^{\bullet}$ as a result of the H-donating ability, which can be analyzed spectrophotometrically at 517 nm. Similarly, ferulic, 3,4-dihydroxyphenyl acetic, dihydrocaffeic acids showed a radical scavenging activity towards the free radical $DPPH^{\bullet}$, however their phenolic lipids ester demonstrated a lower scavenging activity than that of its corresponding phenolic acid, but compared to that of α-tocopherol (Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009).

Thiobarbituric acid reactive substances (TBARS) measure the secondary oxidative products such as malonaldehyde (MDA), which is formed from the decomposition of lipid hydroperoxides. The thiobarbituric acid reacts with MDA to form a colored product with maximum absorption at 532 nm, which can be measured spectrophotometrically (Handelman and Pryor, 1999).

CHAPTER III

STATEMENT OF CHAPTER III LINKAGE

Chapter III involves the development of a model reaction conditions for the enzymatic synthesis of phenolic lipids, from flaxseed oil and selected phenolic acids in solvent-free medium. The production of selected phenolic lipids with enhanced solubility and anti-oxidative properties was as part of ongoing work in our laboratory; however, previous work was conducted in organic solvent media, while the presented one aims to develop a bioprocess for a clean and environmental safe approach. The effects of phenolic acid structure, different surfactants, substrate concentration and reaction time course on the bioconversion yield and productivity were investigated. The novel synthesized phenolic lipids molecular structures were characterized by the APCI-MS analysis.

CHAPTER III

LIPASE-CATALYZED SYNTHESIS OF STRUCTURED PHENOLIC LIPIDS IN SOLVENT-FREE MEDIUM USING FLAXSEED OIL AND SELECTED PHENOLIC ACIDS

3.1. Abstract

Structured phenolic lipids (PLs) were obtained by lipase-catalyzed transesterification of flaxseed oil in a solvent-free medium (SFM), using selected phenolic acids, including hydroxylated and/or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids. A bioconversion yield of 65% was obtained for the transesterification of flaxseed oil with 3,4-dihydroxyphenyl acetic acid (DHPA). In addition, the effect of the chemical structure of phenolic acids on the transesterification of flaxseed oil and selected phenolic acids in SFM was of less magnitude as compared to that in organic solvent media (OSM). Using DHPA, the APCI-MS analysis confirmed the synthesis of monolinolenyl, dilinolenyl, linoleyl linolenyl and oleyl linolenyl dihydroxyphenyl acetates as the phenolic lipids. A significant increase in the enzymatic activity from 200 to 270 nmol of PLs/g solid enzyme/min was obtained upon the addition of the non-ionic surfactant Span 65. However, with the addition of the anionic surfactant Sodium bis-2-ethylhexyl sulfosuccinate (AOT) and the cationic one, hexadecyltrimethylammonium bromide (CTAB), the enzymatic activity was decreased slightly from 200 to 192 and 190 nmol of PLs/g solid enzyme/min, respectively. The results also showed that the increase in DHPA concentration from 20 to 60 mM resulted in a significant increase in the volumetric productivity (P_V) from 1.61 to 4.74 mg PLs per mL reaction mixture per day.

3.2. Introduction

There is a growing interest in the development of nutraceuticals as food supplements as well as natural bio-ingredients to fulfill the high consumer's demand for health-promoting food products. The numerous health benefits of the ω-3 polyunsaturated fatty acids (PUFAs) have been widely recognized in the modulation of risk of a variety of diseases (Roche, 1999). The incorporation of selected PUFAs into the diet is therefore of great importance. Although PUFAs are found in vegetable and fish oils, the formulation of nutraceuticals with ω-3 PUFAs cannot be done without simultaneous incorporation of an acceptable antioxidant as they are prone to

oxidative deterioration, and may lead to the formation of off-flavors and potentially harmful radical products (Ito *et al*., 1986).

On the other hand, synthetic antioxidants being potentially carcinogenic (Shahidi and Naczk, 1995a) are continuously being replaced by natural antioxidants from plant sources, such as phenolic compounds (Torres de Pinedo *et al.,* 2007). In addition to their antioxidant properties, phenolic compounds have also other vital beneficial properties in the biological systems (Silva *et al*., 2000). However, the low solubility of phenolic acids in non-polar media reduces their effectiveness in fat and oil systems (Stamatis *et al*., 2001). The structuring of triacylglycerols with phenolic acids could potentially result in novel structured phenolic lipids (Akoh *et al.,* 1998). The numerous combined beneficial properties of both PUFAs and phenolic compounds can be offered by these novel structured biomolecules, with enhanced anti-oxidative and functional properties (Stamatis *et al*., 2001; Sabally *et al*., 2007).

Research work, carried out recently in our laboratory has succeeded in the enzymatic synthesis of phenolic lipids in organic solvent media (OSM), including lipase-catalyzed esterification of fatty alcohols and phenolic acids (Lue *et al*., 2005) and the transesterification of phenolic acids with acylglycerol models (Sabally *et al.,* 2006a; Safari *et al*., 2006) as well as edible oils, including flaxseed and fish liver oils (Sabally *et al.,* 2006b; Sabally *et al.,* 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). However, a major drawback limiting the enzymatic synthesis of phenolic lipids at a large scale is the low volumetric productivity. In addition, the use of some organic solvents may limit the acceptability of nutraceuticals and food ingredients (Martinez *et al*., 2004). Thus the development of an efficient and cost effective process for the enzymatic synthesis of these phenolic lipids in a large scale is of utmost importance. One of the most promising novel approaches consists of using solvent-free-medium (SFM), which may allow the use of a smaller reaction volume, higher substrate concentrations and avoid the process of solvent-recovery (Dossat *et al.,* 2002a; Fregolente *et al*., 2008). Hence, the development of bioprocess for the lipase-catalyzed synthesis of phenolic lipids in SFM is of major interest.

The overall objective of the present work was to optimize the lipase-catalyzed synthesis of phenolic lipids in SFM, using flaxseed oil and selected phenolic acids as substrates. The specific objectives were to investigate the effects of phenolic acid structure, different surfactants,

substrate concentration and time course on the bioconversion yield and productivity. In addition, the synthesized phenolic lipids molecular structures were characterized by the APCI-MS analysis.

3.3. Materials and Methods

3.3.1. Materials

Commercial immobilized lipase from *Candida antarctica* (Novozym 435, with an activity of 10,000 propyl laurate units, PLU, per g solid enzyme) was obtained from Novozymes Nordisk A/S (Bagsværd, Denmark). Selected phenolic acids, including cinnamic, dihydrocaffeic and 3,4-dihydroxyphenyl acetic, 3,4-dimethoxybenzoic, ferulic and sinapic acids as well as surfactants, including Span 65 (Sorbitan tristearate), AOT (Sodium bis-2-ethylhexyl sulfosuccinate) and CTAB (Hexadecyltrimethylammonium bromide), were purchased from Sigma Chemical Co. (St-Louis, MO). Flaxseed oil was a gift from Arista Industries, Inc. (Wilton, CT). Organic solvents of high-performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific (Fair Lawn, N.J.).

3.3.2. Transesterification Reaction in Solvent-Free Medium

Lipase-catalyzed transesterification of flaxseed oil with selected phenolic acids, including cinnamic, dihydrocaffeic, 3,4-dihydroxyphenyl acetic, 3,4-dimethoxybenzoic, ferulic and sinapic acids was carried out, according to the modification of Sabally *et al.* (2006b) method developed in our laboratory. Prior to each enzymatic reaction, a stock solution of phenolic acid (143 mM) in 2-butanone was freshly prepared. Aliquot of the phenolic acid stock solution was mixed with the flaxseed oil to acquire a final concentration of 10 mM, where 2-butanone was 7% of the total reaction volume. The enzymatic reaction was initiated by the addition of 50 mg of solid enzyme (500 PLU)/mL. The reaction mixture was incubated at 55°C, with continuous shaking at 150 rpm in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The transesterification reactions were run in duplicate in tandem with control trials that contain all components except the enzyme. The enzymatic reaction was monitored at selected time intervals over the course of 10 days. The recovered samples were flushed with a gentle stream of nitrogen and stored at - 80ºC for further analysis.

3.3.3. Characterization of End Products

3.3.3.1. HPLC Analysis of the Reaction Components

HPLC analysis of reaction components was carried out according to the method developed in our laboratory (Sabally *et al.,* 2006b). The separation was performed on an Agilent Zobrax SB-C18 reversed-phase column (250x4.6 mm, 5 µm), using a Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). A volume of 20 µL sample was recovered and solubilized in 150 µL isopropanol. A volume of 20 µL of the diluted sample was subjected to HPLC analysis. The elution of the injected sample was carried out by a gradient solvent system, using acetonitrile/methanol mixture (7:5, v/v) as solvent (A) and isopropanol as solvent (B). The elution was initiated by an isocratic flow of 100% of solvent A for 10 min, followed by a 10 min linear gradient to 40 and 60% of solvent A and B, respectively, then to 100% of solvent B for 10 min period. The elution was maintained for an additional period of 5 min before reverting to the initial conditions (100% solvent A), followed with an equilibration period of 10 min for the next sample. The flow rate was at 1 mL/min and the detection was performed at 280 nm for monitoring the phenolic lipids products.

The maximal bioconversion yield of phenolic lipids was calculated as the total peak area of phenolic lipids, detected at 280 nm, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100. The enzymatic activity was calculated from the slope of the linear portion of the plot of bioconversion yield versus the reaction time and it was expressed as nmol of PLs per g solid enzyme per min of reaction. The bioconversion yield of phenolic monoacylglycerols was defined as the peak area of the phenolic lipids, detected at 280 nm and eluted between 3 and 9 min, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100. However, the bioconversion yield of phenolic diacylglycerols was defined by the peak area of the phenolic lipids, detected at 280 nm and eluted between 10 and 20 min, divided by the peak area of residual phenolic acid and that of total phenolic lipids, multiplied by 100.

3.3.3.2. Mass Spectrometry Analysis of End Products

The characterization of reaction components of the lipase-catalyzed transesterification reaction was also performed by HPLC interfaced to an atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The APCI-MS system (ThermoFinnigan, San Jose, CA) was equipped with the Zorbax SB-C18 reversed-phase column as well as a Surveyor liquid chromatography pump, an autosampler and Xcalibur® system control software (version 1.3) for data acquisition and processing. The mass spectrometer was operated in positive ion mode with a collision energy source of 15 V. The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

3.3.4. Effect of Surfactants on the Bioconversion Yield

The effect of different surfactants, including Span 65, AOT and CTAB, at a range of concentrations from 1 to 5 mM, on the transesterification reaction was investigated.

3.3.5. Effect of Phenolic Acid Concentration on the Bioconversion Yield and Productivity

The effect of 3,4-dihydroxyphenyl acetic acid (DHPA) concentrations (20 to 60 mM) on the bioconversion yield and productivity was carried out, with/without substitution of the lipase by a fresh one after 3 days of the reaction time course.

3.3.6. Statistical Analysis

All experiments were analyzed as completely randomized design using PROC ANOVA of the statistical analysis system (SAS, 2009). Multiple comparisons of mean values were done by Tukey's honest significance test at $P < 0.05$.

3.4. Results and Discussion

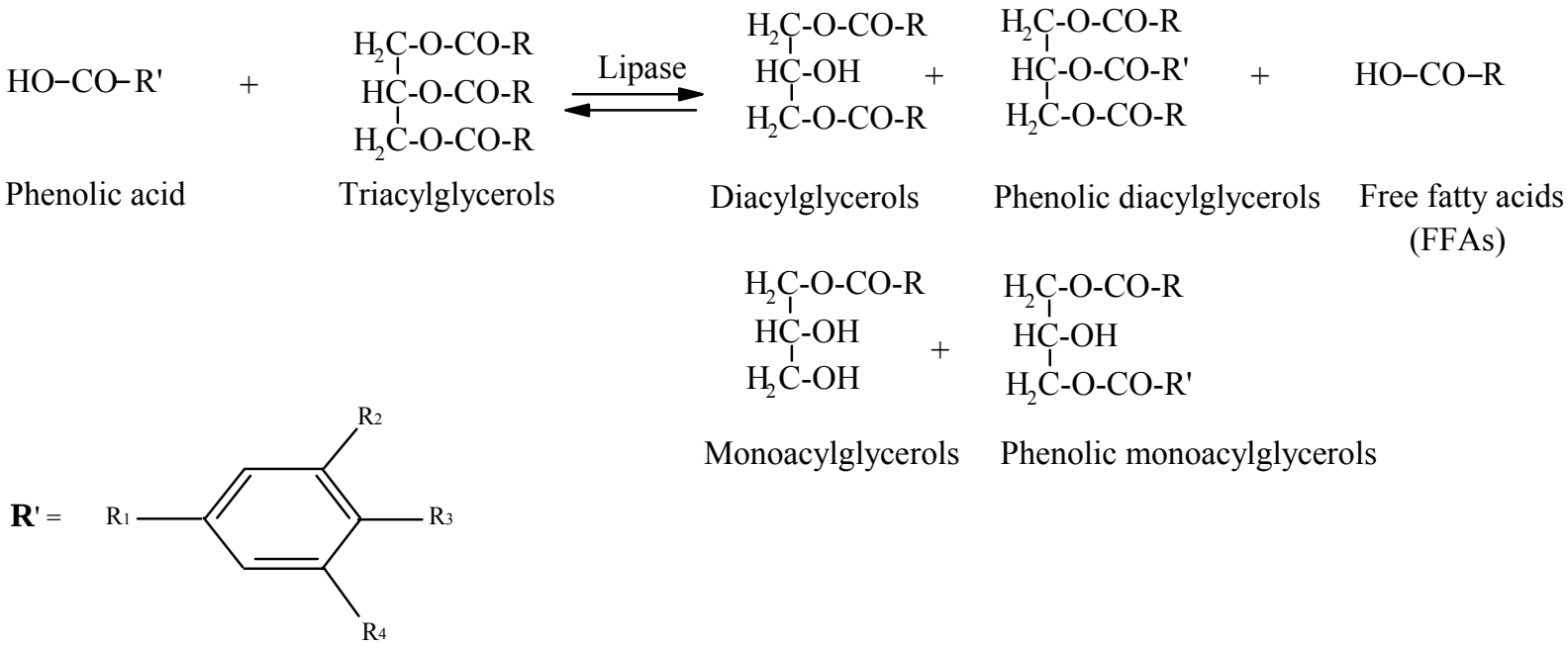
3.4.1. Effect of Phenolic Acid Structure on the Bioconversion Yield

The enzymatic synthesis of phenolic lipids by the transesterification of flaxseed oil with selected phenolic acids in SFM was investigated, using Novozym 435 from *Candida antarctica* as the biocatalyst (Fig. 9). The investigated phenolic acids (Table 3) showed maximum bioconversion yield after 7 days of reaction. However, the bioconversion yield was decreased by 1-13% using selected phenolic acids, including cinnamic, dihydrocaffeic, DHPA, ferulic and sinapic acids after 10 days of reaction; this decrease may be due to a shift in the thermodynamic equilibrium

of the reaction toward the hydrolysis and/or to the acyl migration of the phenolic group (Karboune *et al.,* 2008).

The highest bioconversion yield (87%) was obtained with cinnamic acid after 7th day of reaction; however, the *p*-hydroxylation of its aromatic ring (dihydrocaffeic and DHPA) resulted in a significant decrease ($P < 0.05$) in the bioconversion yield to 58 and 65%, respectively. In addition, the methoxylation of the aromatic ring (sinapic, 3,4-dimethoxybenzoic and ferulic acids) resulted in a significant decrease ($P < 0.05$) in the bioconversion yield to 55, 57 and 49%, respectively. The inhibitory effect of the hydroxyl and methoxyl substituents of cinnamic acid derivatives on the enzyme activity have been reported previously (Buisman *et al*., 1998; Safari *et al*., 2006) and it was attributed to their electronic donating effects and/or to their steric hindrances in the enzyme active site. In addition, Table 3 shows that the use of dihydrocaffeic and 3,4-dimethoxybenzoic acids as substrates resulted in similar bioconversion yield (~57%), the overall results suggest that the effect of methoxyl and hydroxyl groups on the enzyme activity in SFM are somehow comparable with no significant difference ($P > 0.05$). The lack of a double bond in the side-chain, conjugated with the aromatic ring of DHPA, may decrease the inhibitory effect of the *p*-hydroxylation of the aromatic ring, where 65% of bioconversion yield was obtained. These findings suggest that the inhibitory effect of *p*-hydroxyl substituent may be due to the electronic donating effect rather than to the steric hindrance (Buisman *et al*., 1998). Similarly, Karboune *et al.* (2008) reported that the inhibitory effect of *p*-hydroxyl substituents was probably due to their electronic donating effects, which change the electronic configuration of the phenolic acid creating unstable substrates for the enzyme. The overall results (Table 3) indicate that the bioconversion yield of phenolic lipids from flaxseed oil in SFM was slightly dependent on the structural characteristics of phenolic acids.

In contrast to the use of SFM as a reaction medium, a significant effect of the chemical structure of different phenolic acids on the bioconversion yield was reported in OSM for the transesterification of flaxseed oil with selected phenolic acids (Karboune *et al.,* 2008); such variations between the two media may be attributed to their differences in polarity and viscosity (Karboune *et al.,* 2005). The hydrophobic SFM medium containing flaxseed oil may have increased the structural rigidity of lipase conformation, as compared to OSM, which could result in a less effect of the phenolic acid structures (Fitzpatrick and Klibanov, 1991; Dordick, 1992).



R = Flax seed oil FFAs ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$)

Cinnamic acid : R_1 = CH = CH, R_2 = H, R_3 = H, R_4 = H
Dihydrocaffeic acid : R_1 = CH_2-CH_2, R_2 = H, R_3 = OH, R_4 = OH
3,4-Dihydroxyphenyl acetic acid : R_1 = CH_2, R_2 = H, R_3 = OH, R_4 = OH
3,4-Dimethoxybenzoic acid : R_1 = H, R_2 = H, R_3 = O-CH_3, R_4 = O-CH_3
Ferulic acid : R_1 = CH = CH, R_2 = H, R_3 = OH, R_4 = O-CH_3
Sinapic acid : R_1 = CH = CH, R_2 = O-CH_3, R_3 = OH, R_4 = O-CH_3

Figure 9. Reaction scheme of lipase-catalyzed transesterification reactions of selected phenolic acids with flaxseed oil.

Table 3. Effect of phenolic acid structure on the bioconversion yield of lipase-catalyzed transesterification of flaxseed oil with selected phenolic acids in solvent-free medium.

Phenolic compound	Total bioconversion yield (%)[a]	
	Day 7[b]	Day 10[b]
Cinnamic acid	86.5 (0.2)[c]	80.4 (0.4)[c]
Dihydrocaffeic acid	57.5 (2.2)[c]	53.7 (3.3)[c]
3,4-Dihydroxyphenyl acetic acid	65.4 (0.1)[c]	58.0 (0.7)[c]
3,4-Dimethoxybenzoic acid	56.6 (2.4)[c]	56.5 (2.9)[c]
Ferulic acid	48.6 (4.1)[c]	35.3 (2.2)[c]
Sinapic acid	55.0 (2.4)[c]	54.3 (0.1)[c]

[a]Total bioconversion yield was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b]Transesterification reaction of flaxseed oil with selected phenolic acids was carried out in solvent-free medium at 55ºC for a reaction period of 7 and 10 days.

[c]Data are average of duplicate samples, where the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

3.4.2. Effects of Solvent Concentration and Time Course of Reaction

In order to minimize the amount of 2-butanone used for the solubilization of DHPA, different solvent concentrations, including 5, 7 and 10% of the total reaction volume, were investigated. Table 4 shows that the highest enzymatic activity (196 nmol of PLs/g solid enzyme/min) and a bioconversion yield of 61% were obtained with the use of 7% of 2-butanone. On the basis of these experimental findings, 7% of 2-butanone was selected as the appropriate solvent concentration.

The time course (Fig. 10) for the enzymatic synthesis of selected phenolic lipids, over a 9-day period of lipase-catalyzed transesterification of flaxseed oil with two selected phenolic acids, DHPA and dihydrocaffeic acid (DHCA), was investigated. The overall results show that the enzymatic synthesis of 3,4-dihydroxyphenyl acetoylated and dihydrocaffeoylated lipids were initiated rapidly within the first day of reaction to reach a bioconversion yield of 40 and 34%, respectively. In contrast, the time course for the transesterification reactions of phenolic acids with triolein (Safari *et al*., 2006) and with flaxseed oil (Karboune *et al.,* 2008) in OSM showed an elapsing period of 1 day of reaction. The difference in the time course profile between the OSM and the SFM may be due to the variation in the transesterification/hydrolysis extent ratio. Figure 10 also shows that the highest bioconversion yields of 3,4-dihydroxyphenyl acetoylated and dihydrocaffeoylated lipids of 63 and 57%, respectively, were obtained after 7 days of reaction, however, there was a decrease in the bioconversion yield after 2 additional days to 52 and 49%, respectively. These findings suggest that the reaction carried out in SFM reached its thermodynamics equilibrium faster than that in OSM, where the maximum bioconversion yield (66%) was obtained at day 9 before it was decreased to 55% at day 10 (Karboune *et al.,* 2008). Although the time course for both investigated phenolic acids have the same pattern, the extent of bioconversion was higher with DHPA than that with DHCA. In addition, Figure 10 shows that the trend of the enzymatic synthesis for 3,4-dihydroxyphenyl acetoylated mono- and diacylglycerols were similar to that for dihydrocaffeoylated mono- and diacylglycerols throughout the 9-days reaction period. The bioconversion yield of 3,4-dihydroxyphenyl acetoylated and dihydrocaffeoylated monoacylglycerols increased slightly up to a maximum value of 7 and 6%, respectively. While the bioconversion yield of 3,4-dihydroxyphenyl acetoylated and dihydrocaffeoylated diacylglycerols increased rapidly within the first 3 days of

Table 4. Effect of 2-butanone on the bioconversion yield of phenolic lipids.

	2-butanone (%)		
	5%	7%	10%
Total bioconversion yield (%)[a]	47.6 (3.9)[c]	61.1 (6.1)[c]	56.2 (1.5)[c]
Enzyme activity[b]	136.6 (6.4)[c]	195.6 (4.4)[c]	191.5 (7.0)[c]

[a]Total bioconversion yield was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b]Enzyme activity was defined as nmol of PLs/g solid enzyme/min reaction time.

[c]Data are average of duplicate samples, where the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

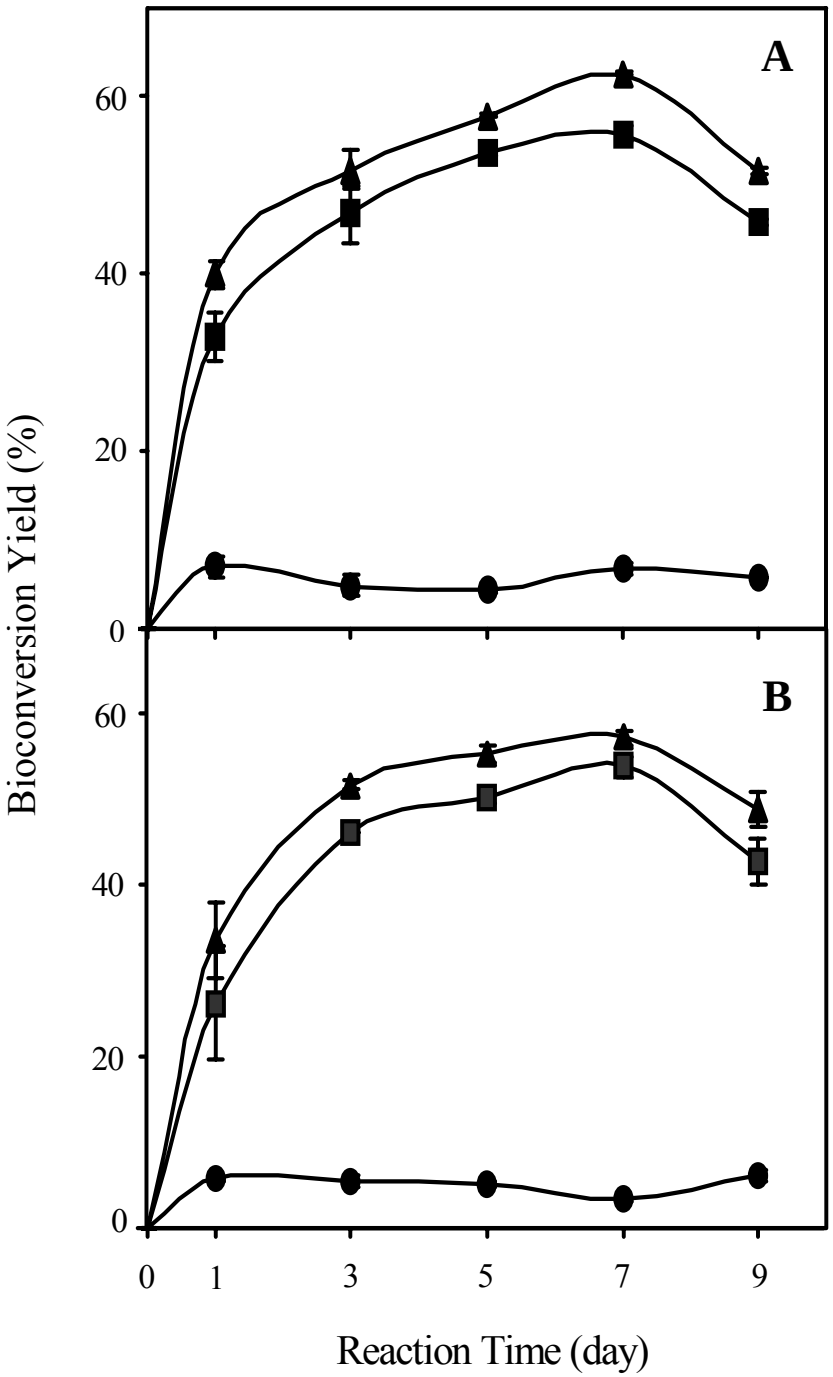


Figure 10. Time course of transesterification reaction using 3,4-dihydroxyphenyl acetic acid (A) and dihydrocaffeic acid (B) with flaxseed oil in solvent-free medium showing total phenolic lipids (▲), phenolic monoacylglycerols (●) and phenolic diacylglycerols (■), STD ≤ 6.5.

reaction to 47 and 46%, respectively, and to a lower extent thereafter up to a maximum of 56 and 54% after 7 days of reaction. However, the bioconversion yield decreased to 46 and 43%, respectively, after 2 additional days of reaction. This decrease may be due to a shift in the thermodynamic equilibrium of the reaction toward hydrolysis and/or to the acyl migration, as a result of increase in the concentration of free fatty acids (Karboune *et al.,* 2005).

In contrast to the OSM (Karboune *et al.,* 2008), lipase-catalyzed transesterification of flaxseed oil with selected phenolic acids in SFM was more in favor of the synthesis of phenolic diacylglycerols. The biocatalysis in OSM (Karboune *et al.,* 2008) showed a maximum bioconversion yield of 3,4-dihydroxyphenyl acetoylated mono- and diacylglycerols of 37 and 29%, respectively, whereas the use of SFM (Fig. 10A) resulted in a bioconversion yield of 7 and 56%, respectively. The difference in reaction selectivity between the OSM and SFM may be attributed to their micro-environment effect affecting differently the substrate availability (Karboune *et al.,* 2005).

The micro-environment of the biocatalyst consists of a thin layer of surrounding water, which forms the transition between the biocatalyst and the reaction medium; this layer is tightly bound to the biocatalyst, acting as a buffer between the enzyme surface and the reaction medium (Pires-Cabral *et al.,* 2005). The polarity of the reaction medium is an important parameter because of its ability to modify the partition of water, substrates and products between the solid phase of the enzyme and the liquid phase, resulting in changes in the enzyme activity and selectivity (Fukui *et al*., 1987; Laane, 1987). In SFM, the presence of excess of flaxseed oil increases the interaction between the oil and the enzyme, resulting in an increase in the concentrations of mono and diacylglycerols in the reaction medium, thus favoring the enzymatic synthesis of phenolic lipids (Sabally *et al*., 2006b). The polarity and the viscosity of the reaction medium may also have affected the substrate availability and hence the bioconversion rate (Dossat *et al.,* 2002b; Wei *et al.,* 2003). The availability of the less polar substrate diacylglycerols was increased favoring the production of the more hydrophobic substrate, which was the phenolic diacylglycerols (Sabally *et al*., 2006b). Bellot *et al.* (2001) indicated that the polarity of the reaction medium has a great effect on the selectivity of the lipase-catalyzed esterification reaction between glycerol and oleic acid.

Karboune *et al*. (2005) reported that an excess of triolein in OSM favored the formation of dioleyl cinnamate than that of monooleyl cinnamate in lipase-catalyzed synthesis of cinnamoylated lipids, hence altering the reaction selectivity. Such a phenomenon has also been reported for the lipozyme-catalyzed transesterification of sunflower oil with butanol, whereby a change in the polarity of the micro-environment of the enzyme may have affected the availability of substrates and products (Dossat *et al*., 2002b).

Each enzyme has specific water activity (a_w) requirements (Halling, 1994). Various features of reaction selectivity of lipases are modulated by exogenous factors, such as the type of organic solvent, choice of co-substrates, a_w, pH and immobilization (Lee and Parkin, 2001). Broos *et al.* (1995) reported that an increase in the lipase flexibility was correlated with a higher enzyme enantioselectivity. Different responses of lipase selectivity at various a_w may be related to changes in enzyme conformational flexibility of active sites (Affleck *et al.,* 1992; Klibanov, 1997) as well as to differences in primary structure known to exist in substrate binding domains for the enzyme (Kazlauskas, 1994). Alternatively, the presence of micro-environments in heterogeneous systems may influence the enzyme selectivity by affecting differentially partitioning behavior of the substrate in the reaction mixture (Halling, 1994; Pencreach and Baratti, 1997). The effect of a_w on the positional selectivity of the immobilized lipase Novozym 435 during the esterification of oleic acid with glycerol was investigated, and the highest preferential selectivity of Novozym 435 to 1-position over 2-position of the glycerol molecule was achieved at a_w of 0.53 (Duan *et al.,* 2010).

3.4.3. Structural Characterization of Phenolic Lipids

Figure 11 shows the HPLC elution profile for the reaction components of lipase-catalyzed transesterification reactions of flaxseed oil with DHPA and DHCA, monitored at 280 nm. Peak #1 (Figs. 11A and 11B) and 1' (Figs. 11A' and 11B'), were characterized as DHPA and DHCA, respectively. Four predominant peaks (3, 4, 5 and 6) were characterized as the main phenolic lipids obtained by the transesterification reaction using flaxseed oil and DHPA, since they have shown a UV-spectral scanning profile comparable to that of its phenolic acid component (Peak #1). Similarly, peaks (2', 5', 6' and 7') were characterized as the main phenolic lipids obtained by the transesterification reaction of flaxseed oil and DHCA. TAGs of flaxseed oil were

identified as peaks #8 to 13 and #8' to 13' in the elution profile of the reaction components obtained with DHPA (Figs. 11A and 11B) and DHCA (Figs. 11A' and 11B'), respectively.

In order to characterize the molecular structure of phenolic lipids, further analysis by HPLC/APCI-MS spectrometry of the eluted peaks was conducted. The phenolic group substitution on the glycerol backbone of the phenolic lipids is shown for simplicity, as the regioselectivity of the reaction is unknown (Karboune *et al.,* 2008). The fragmentation patterns of the main phenolic lipid products, using DHPA, are shown in Figure 12; the fragmentation of peak #3 (Fig. 11B) produced a fragment ion at *m/z* 335.3, representing monolinolenin as well as abundant molecular ions at *m/z* 503.3 $[M+H]^+$ and 485.6 $[M+H-H_2O]^+$, corresponding to monolinolenyl 3,4-dihydroxyphenyl acetate (Fig. 12A). The results (Fig. 12B) also indicate that the fragmentation pattern of peak #4 (Fig. 11B) has a major molecular ion $[M]^+$ at *m/z* 763.7, corresponding to dilinolenyl 3,4-dihydroxyphenyl acetate. Peak #5 (Fig. 11B) was characterized as linoleyl linolenyl 3,4-dihydroxyphenyl acetate, with an abundant molecular ion $[M]^+$ at *m/z* 765.6 and other fragment ions at *m/z* 335.4, 337.5 and 615.6, corresponding to monolinolenin $[M+H-H_2O]^+$, monolinolein $[M+H-H_2O]^+$ and linoleyl linolenyl glycerol $[M+H]^+$, respectively (Fig. 12C). While the fragmentation of peak #6 (Fig. 11B) resulted in a characteristic pattern of oleyl linolenyl 3,4-dihydroxyphenyl acetate (Fig. 12D), with an abundant molecular ion at *m/z* of 767.5 $[M]^+$ and fragment ions at 335.3, 339.4 and 617.6, corresponding to monolinolenin $[M+H-H_2O]^+$, monoolein $[M+H-H_2O]^+$ and oleyl linolenyl glycerol $[M+H]^+$, respectively.

In addition, the fragmentation patterns of the main phenolic lipid products, using DHCA, are shown in Figure 12, where the dihydrocaffeoyl group substitution at the glycerol backbone of the phenolic lipids is shown for simplicity as the regioselectivity of the reaction is unknown (Karboune *et al.,* 2008). The fragmentation pattern of peak #2' (Fig. 11B') shows a molecular ion of *m/z* 517.3 $[M+H]^+$, corresponding to monolinolenyl dihydrocaffeate and a fragmentation of *m/z* 335.3 $[M+H-H_2O]^+$ representing monolinolenin (Fig. 12A'). Fragmentation of peak #5' (Fig. 11B') produced an abundant molecular ion $[M+H]^+$ of *m/z* 777.6 characteristics of dilinolenyl dihydrocaffeate (Fig. 12B') and fragment ions of *m/z* 595.5, 499.4 and 335.3, corresponding to dilinolenin $[M+H-H_2O]^+$, monolinolenyl dihydrocaffeate $[M+H-H_2O]^+$ and monolinolenin $[M+H-H_2O]^+$, respectively.

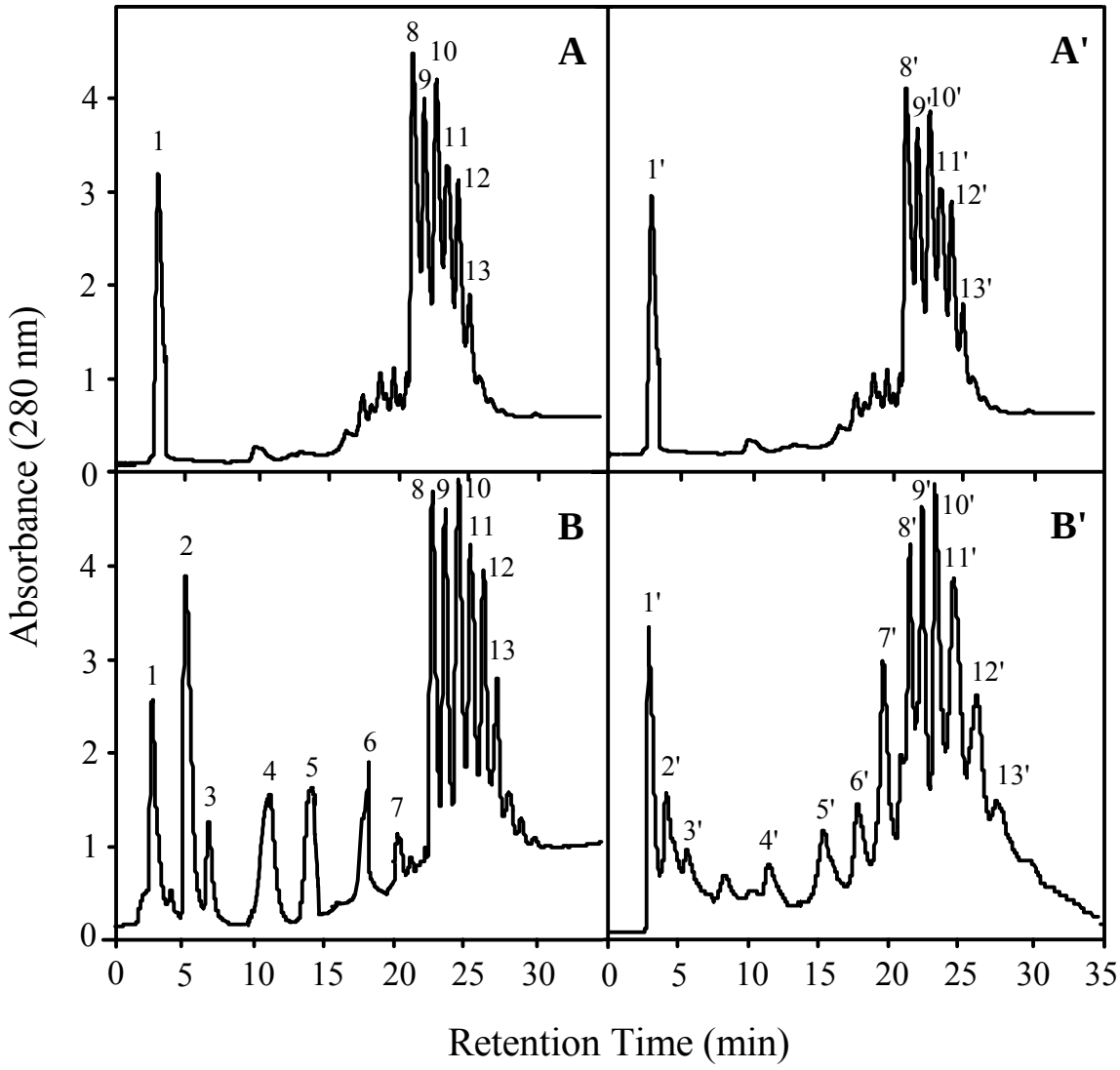


Figure 11. HPLC chromatograms of the initial reaction mixture of 3,4-dihydroxyphenyl acetic and dihydrocaffeic acids with flaxseed oil (A and A'), respectively, and the enzymatic transesterification reaction mixture (B and B') monitored at 280 nm.

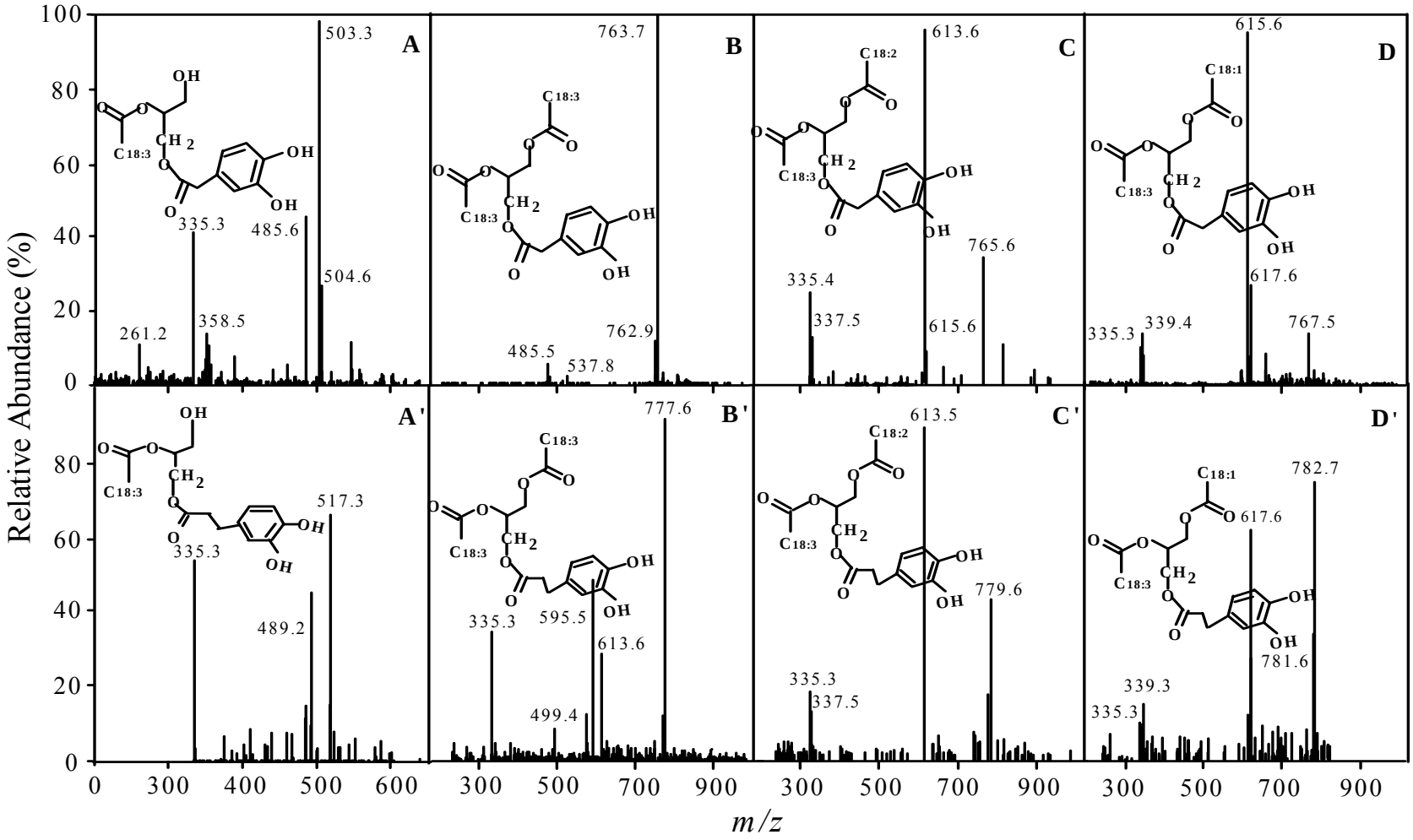


Figure 12. Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) analysis of 3,4-dihydroxyphenyl acetoylated monoacylglycerols, -diacylglycerols (A, B, C and D) and dihydrocaffeoylated monoacylglycerols, -diacylglycerols (A', B', C' and D').

Fragmentation of peak #6' (Fig. 11B') resulted in a mixed phenolic diacylglycerol molecular ion of *m*/*z* 779.6 $[M+H]^+$ being linoleyl linolenyl dihydrocaffeate with fragment ions of *m*/*z* 337.5 and 335.3, corresponding to monolinolein and monolinolenin, respectively (Fig. 12C'). While fragmentation of peak #7' (Fig. 11B') resulted in molecular ion of *m*/*z* 782.7 $[M+H]^+$ being oleyl linolenyl dihydrocaffeate with fragment ions of *m*/*z* 339.3 and 335.3, corresponding to monoolein and monolinolenin, respectively (Fig. 12D'). The loss of H_2O from a monoacylglycerol during HPLC/APCI-MS analysis has been previously reported (Sabally *et al.,* 2006b; Safari *et al.,* 2006; Karboune *et al*., 2008; Karam *et al.,* 2009).

Although, the HPLC/APCI-MS analysis confirmed the formation of various phenolic mono- and diacylglycerols, by lipase-catalyzed transesterification of flaxseed oil with DHPA and DHCA in SFM; phenolic diacylglycerols were the main synthesized phenolic lipids. Likewise, Sabally *et al.* (2006b) and Karboune *et al.* (2008) have characterized 6 dihydrocaffeoylated lipids (monolinolenyl, monooleyl, dilinoleyl, dilinolenyl, oleyl linolenyl and dioleyl dihydrocaffeates), as well as 6 dihydroxyphenyl acetoylated lipids (monolinolenyl, monolinoleyl, dilinolenyl, dilinoleyl, linoleyl linolenyl and oleyl linolenyl 3,4-dihydroxyphenyl acetates) as the end products of lipase-catalyzed transesterification of flaxseed oil in OSM, with DHCA and DHPA, respectively, as substrates.

3.4.4. Effect of Surfactants on the Bioconversion Yield of Phenolic Lipids

Surfactants have the ability to decrease the surface tension and, as a result, increase the interfacial area between substrates and enzyme molecules; such effects would presumably harbor more lipase molecules, where the increase in the solubility of substrates would consequently increase the rate of the enzymatic reaction (Liu *et al*., 2000). Selected surfactants including non-ionic (Span 65), cationic (CTAB) and anionic (AOT), were added separately to the reaction medium. The effects of these surfactants on the enzyme activity and the bioconversion yield of phenolic lipids in SFM were investigated. The results (Table 5) indicate that the use of Span 65 resulted in a slightly higher bioconversion yield of 67% and a significant increase ($P < 0.05$) in the enzyme activity of 270 nmol of PLs/g solid enzyme/min as compared to the control without surfactant (63% and 200 nmol of PLs/g solid enzyme/min). While, the ionic surfactants AOT and CTAB slightly decreased the bioconversion yield of phenolic lipids to 56 and 58%, respectively, and the enzyme activity to (~190 nmol of PLs/g solid enzyme/min). In contrast, Liu *et al*. (2000)

reported a higher enzymatic activity in OSM, up to 13 times for *Candida rugosa* lipase, coated with the non-ionic surfactant Tween-80, as compared to the one coated with anionic or cationic surfactants. The literature (Xia *et al.,* 1996; Liu *et al*., 2000) indicates that the enhancement of *Penicillium cyclopium* and *Candida rugosa* lipases catalytic activity was dependent on the type of surfactants. The reduced enzyme activity in the presence of ionic surfactants could be attributed to the presence of strong interaction between the cationic or anionic head group in the surfactant molecule and the negatively charged lipase, which may induce a change in the three dimensional structure of lipase (Kamiya *et al.,* 1995; Liu *et al*., 2000). In addition, it was suggested (Goto *et al.,* 1994) that the interaction between the non-ionic surfactant and the lipase via a weak interaction, would favor the maintenance of the stereoconfiguration of lipase for better catalytic performance, which would result in higher bioconversion yield.

In conclusion, the effect of surfactant addition on the bioconversion yield was not highly significant in SFM (Table 5) as compared to performing the reaction in OSM or buffer medium (Kamiya *et al.,* 1995; Goto *et al.,* 1994; Liu *et al*., 2000), this result may be attributed to the nature of the SFM in terms of viscosity and polarity, which would have affected the interactions between the lipase and the surfactants. The hydrophobic SFM medium containing flaxseed oil may have increased the structural rigidity of lipase conformation, as compared to OSM, which could have result in a less effect of the surfactants (Fitzpatrick and Klibanov, 1991; Dordick, 1992).

3.4.5. Effect of Phenolic Acid Concentration on the Bioconversion Yield and Productivity

In order to maximize the bioconversion yield, different phenolic acid concentrations, including 20, 40 and 60 mM of DHPA, were investigated (Fig. 13). Using 20, 40 and 60 mM DHPA, the overall time course, showed a significant increase in the total bioconversion yield of phenolic lipids within the first day of reaction up to 49, 45 and 43%, then to a lower extent to reach a maximum value of 66, 67 and 56%, respectively, after 7 days of the enzymatic reaction. However, the bioconversion yield (67%) remained relatively constant when DHPA concentration was increased from 20 to 40 mM. In contrast, increasing the DHPA concentration from 20 to 60 mM resulted in a decrease in the bioconversion yield from 66 to 56% after 7 days of the enzymatic reaction. Such decrease may be due to the enzyme inhibition by the excess of the phenolic acid.

Table 5. Effect of different surfactants on the enzyme activity and the bioconversion yield of lipase-catalyzed transesterification of flaxseed oil with 3, 4-dihydroxyphenyl acetic acid in solvent-free medium.

		Maximum bioconversion yield (%)[a]		
Surfactant[b]	Enzyme activity[c]	Phenolic monoacylglycerols	Phenolic diacylglycerols	Total phenolic lipids
AOT	192	3.6 (1.5)[d]	53.3 (1.6)[d]	55.5 (3.5)[d]
CTAB	190	3.5 (7.0)[d]	54.3 (4.2)[d]	57.8 (2.5)[d]
Span 65	270	7.3 (10.6)[d]	59.1 (1.0)[d]	66.5 (0.2)[d]
Control[e]	200	6.3 (5.4)[d]	56.3 (7.2)[d]	62.6 (0.8)[d]

[a]The percent of maximum bioconversion yield was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b]Transesterification reaction of flaxseed oil and DHPA was carried out in solvent-free medium, in the presence of surfactants, including AOT (1 mM), CTAB (1 mM) and Span 65 (5 mM) for 7 days reaction period.

[c]Enzyme activity was defined as nmol of PLs/g solid enzyme/min reaction time.

[d]Data are average of duplicate samples, where the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

[e]Control trial, without surfactant.

Similarly, Lee and Akoh (1998) reported that increasing caprylic acid concentration in its transesterification reaction with peanut oil resulted in an inhibition in the lipozyme activity, which may be due to the acid inhibitory effect. The literature (Dossat *et al*., 2002b; Yadav and Lathi, 2003) indicated that some lipase-catalyzed esterification or transesterification reactions were inhibited by an excess of alcohol or acid or by both substrates.

The limitation in the enzymatic synthesis of phenolic lipids may be attributed to the enzyme denaturation, and/or to the formation of a hydrophilic hindrance layer of the glycerol by-product of the reaction in the micro-environment of the enzyme, which may increase the substrate diffusional limitations (Karboune *et al*., 2005). The enzyme denaturation was examined by the substitution of the solid enzyme in the reaction mixture by a fresh one after 3 days of lipase-catalyzed transesterification reaction. Using 20 mM DHPA, the substitution resulted in a 22% increase in the maximum bioconversion yield of total phenolic lipids, after an additional 2 days of reaction; these results suggest that the limited increase in the bioconversion yield (Fig. 13) may be partially due to an enzyme denaturation, which is in agreement with results reported by Karboune *et al.* (2005). On the other hand, the productivity of phenolic lipids was estimated to assess the efficiency of lipase-catalyzed transesterification reaction. The mass productivity (P_M) was defined as the weight (mg) of produced PLs per g of initial substrates per day, the enzymatic productivity (P_E) was defined as the weight (mg) of produced PLs per mU of Novozym 435 per day, whereas the volumetric productivity (P_V) was calculated by the weight (mg) of produced PLs per mL of reaction mixture per day.

The effect of DHPA concentration ranging from 20 to 60 mM on the P_M, P_E and P_V, was investigated after the substitution of the solid enzyme in the reaction mixture by a fresh one after 3 days of reaction. The results (Table 6) show that the productivities, P_M, P_E and P_V, were increased significantly ($P < 0.05$) from 1.81, 6.45 and 1.61, respectively, to 5.32, 18.95 and 4.74, respectively, when the DHPA concentration was increased from 20 to 60 mM. It has been reported that in a SFM, the change in the medium polarity influences the substrate and product availability (Bellot *et al.*, 2001; Dossat *et al*., 2002b). Hence, the increase in the DHPA concentration may favor the productivity of phenolic lipids by affecting the thermodynamic equilibrium of the overall reaction towards the synthesis of phenolic lipids.

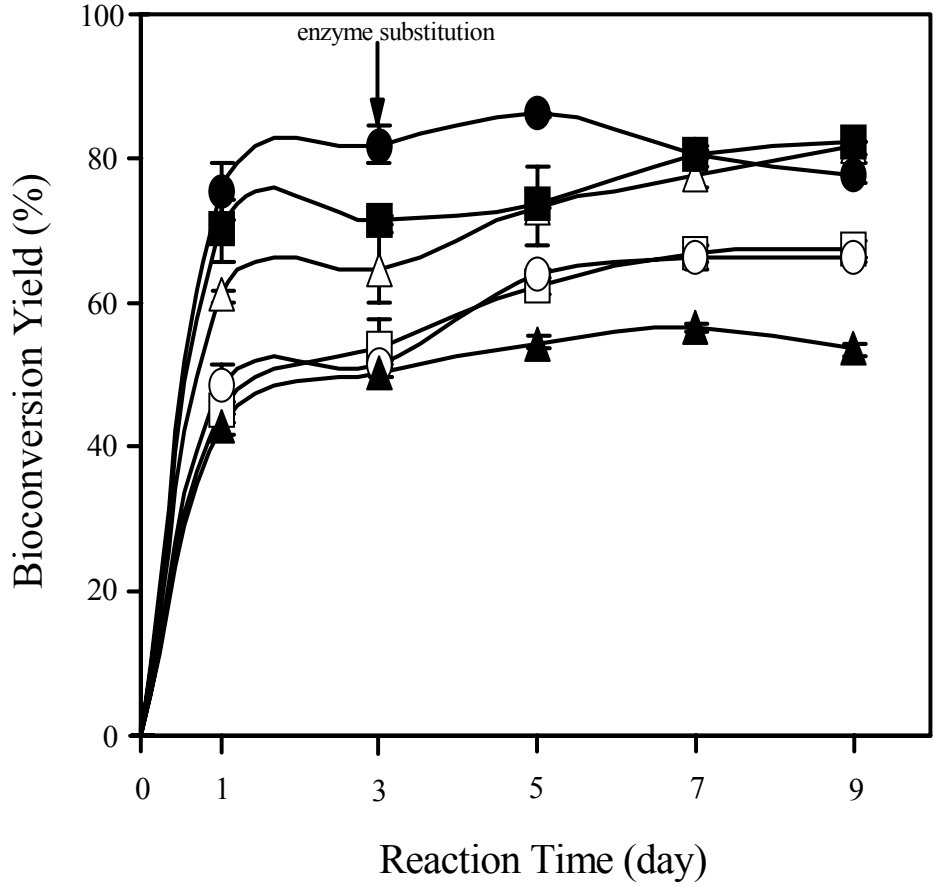


Figure 13. Time course for the transesterification reaction using 3,4-dihydroxyphenyl acetic acid and flaxseed oil in solvent-free medium with different phenolic acid concentrations, 20 mM with (●)/without (o) enzyme substitution at day 3 of the reaction, 40 mM with (■)/without (□) enzyme substitution and 60 mM with (▲)/without (△) enzyme substitution, STD ≤ 5.4.

Table 6. Effect of phenolic acid concentration on the productivities and bioconversion yield of lipase-catalyzed transesterification reaction of 3, 4-dihydroxyphenyl acetic acid with flaxseed oil in solvent-free medium[a].

Phenolic acid (mM)	Mass productivity (mg PLs/g oil/day)[b]	Enzymatic productivity (mg PLs/ mU E/day)[c]	Volumetric productivity (mg PLs/mL/day)[d]	Bioconversion yield (%)[e]
20	1.81 (1.1)[f]	6.45 (2.3)[f]	1.61 (1.4)[f]	80.7 (1.6)[f]
40	3.63 (0.4)[f]	12.92 (3.1)[f]	3.23 (2.4)[f]	80.9 (0.4)[f]
60	5.32 (0.8)[f]	18.95 (5.9)[f]	4.74 (1.6)[f]	79.0 (0.7)[f]

[a]Shows the productivities of 3,4-dihydroxyphenyl acetoylated phenolic lipids (PLs) at day 7.

[b]Mass productivity was calculated on the basis of weight in mg of phenolic lipids per g oil per day of reaction.

[c]Enzymatic productivity was calculated on the basis of weight in mg of phenolic lipids per mU Enzyme per day of reaction.

[d]Volumetric productivity was calculated on the basis of weight in mg of phenolic lipids per mL reaction mixture per day.

[e]Bioconversion yield percent was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[f]Data are average of duplicate samples, and the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

In addition, increasing the DHPA from 20 to 40 mM resulted in a 2-fold increase in the productivity. The linear increase in $P_{M,}$ P_E and P_V values may be due to an increase in the substrate availability, as DHPA concentration increased this might have favored the thermodynamic equilibrium towards the synthesis of phenolic lipids. However, the limited increase in $P_{M,}$ P_E and P_V at a higher DHPA concentration (60 mM) may be due to the denaturation of the enzyme (Yadav and Lathi, 2003), by reactant and/or product inhibition (Garcia *et al.*, 1999). Such limited increase in the productivity also might be explained by the formation of a hydrophilic hindrance layer of glycerol in the micro-environment of the enzyme, which can increase the substrate diffusion limitations (Karboune *et al.*, 2005; Wei *et al*., 2008). The volumetric productivity of the transesterification of DHPA with flaxseed oil in OSM (Karboune *et al*., 2008) was decreased 11-fold using 5 mM DHPA at a substrate molar ratio of 1:8 as compared to that in SFM using 10 mM DHPA. Similarly, Dossat *et al.* (2002a) compared the enzymatic transesterification of high oleic sunflower oil with butanol by the immobilized Lipozyme IM in *n*-hexane and in SFM. The use of SFM resulted in a 6-fold increase in volumetric productivity as compared to the use of *n*-hexane as a reaction medium.

3.5. Conclusion

An efficient process for the enzymatic synthesis of selected phenolic lipids, with high productivity, was achieved using the immobilized *Candida antarctica* lipase (Novozym 435) in SFM. In addition, the use of such medium favored the synthesis of phenolic diacylglycerols as compared to phenolic monoacylglycerols. The investigated approach is a promising and environmentally safe route for the production of structured lipids with phenolic acid moiety.

CHAPTER IV

STATEMENT OF CHAPTER IV LINKAGE

An efficient process for the enzymatic synthesis of selected phenolic lipids, with high productivity, was achieved in Chapter III. As a result, Chapter IV involves the optimization of the enzymatic synthesis of phenolic lipids from flaxseed oil, using 3,4-dihydroxyphenyl acetic acid, which was selected as the appropriate substrate with the highest bioconversion yield (Chapter III). Selected parameters which affect the enzymatic synthesis were optimized, including water activity, enzyme concentration and agitation speed. The molecular structures of the novel synthesized phenolic lipids were characterized and their free radical scavenging activity was also determined, and compared to that of α-tocopherol.

CHAPTER IV

ENZYMATIC SYNTHESIS OF PHENOLIC LIPIDS IN SOLVENT-FREE MEDIUM USING FLAXSEED OIL AND 3,4-DIHYDROXYPHENYL ACETIC ACID

4.1. Abstract

The enzymatic synthesis of phenolic lipids (PLs) by transesterification of flaxseed oil with 3,4-dihydroxyphenyl acetic acid (DHPA) was investigated in solvent-free medium (SFM), using Novozym 435 from *Candida antarctica* as the biocatalyst. The reaction was optimized in terms of water activity, enzyme concentration and agitation speed. Increasing the water activity of the reaction mixture from 0.18 to 0.38 resulted in a significant increase in the bioconversion yield from 62 to 77%. APCI-MS analysis confirmed the formation of six 3,4-dihydroxyphenyl acetoylated lipids, which were monolinolenyl, dioleyl, dilinolenyl, linoleyl linolenyl, oleyl linolenyl and oleyl linoleyl dihydroxyphenyl acetates. The highest enzymatic activity (178 nmol of PLs/g solid enzyme/min) was obtained using 40 mg of solid enzyme (400 PLU)/mL at agitation speed 150 rpm, whereas under the optimized conditions, the phenolic lipids showed a significant increase in the relative proportion of linolenic acid ($C_{18:3}$ *n*-3) that increased from 57% in the flaxseed oil to 75 and 64% in the produced phenolic mono- and diacylglycerols, respectively. In addition, the synthesized phenolic lipids demonstrated radical scavenging activity, expressed as IC_{50} 3.7-fold higher than that of DHPA, but compared to that of α-tocopherol.

4.2. Introduction

In the last decade, the production of "structured lipids" (SLs) with enhanced functional properties has greatly increased. SLs consisting of modified triacylglycerols (TAGs) can be obtained by the incorporation of fatty acids or, by region restructured distribution of fatty acids on the glycerol backbone (Akoh *et al.,* 1998). Phenolic lipids are structured lipids that possess a polyunsaturated hydrocarbon chain and an aromatic ring bearing one or more hydroxyl or methoxyl substitutes, attached via an ester bond (Lue *et al.,* 2005), these novel structured molecules may offer numerous combined beneficial properties of both PUFAs and phenolic compounds with enhanced anti-oxidative and functional properties (Sabally *et al.,* 2006b). The modified lipids with novel properties are of great importance in medical, nutraceutical and food

applications (Humeau *et al*., 1995; Guyot *et al.,* 1997; Stamatis *et al.,* 1999; Osório *et al.,* 2009). The enzymatic synthesis of phenolic lipids could be carried out through lipase-catalyzed incorporation of phenolic acids into TAGs, these enzymatic reactions can be carried out under mild conditions and offers a high selectivity (Humeau *et al*., 1995; Guyot *et al.,* 1997; Stamatis *et al.,* 1999).

Research work, carried out previously, in our laboratory has succeeded in the enzymatic synthesis of selected structured phenolic lipids in organic solvent media (OSM), with enhanced anti-oxidative and solubility properties (Lue *et al*., 2005; Sabally *et al.,* 2006 a,b; Safari *et al*., 2006; Sabally *et al.,* 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). However, a major drawback limiting the enzymatic synthesis of phenolic lipids at a large scale is the low volumetric productivity, besides the use of some organic solvents may limit the acceptability of nutraceuticals and food ingredients (Martinez *et al*., 2004). Consequently, enzyme-mediated direct esterification and transesterification in solvent-free medium (SFM) has received tremendous attention in recent years especially in the food industry; and the development of a bioprocess for the lipase-catalyzed synthesis in SFM is of major interest (Martinez *et al*., 2004).

In our previous initial work (Chapter III), selected phenolic lipids were obtained by the incorporation of selected phenolic acids, including dihydrocaffeic and 3,4-dihydroxyphenyl acetic, 3,4-dimethoxybenzoic, ferulic and sinapic acids into flaxseed oil, in a friendly-environmental process. A high bioconversion yield of 65%, was obtained with 3,4-dihydroxyphenyl acetic acid (DHPA), therefore it was selected as the appropriate substrate. The volumetric productivity in SFM was increased 11-fold as compared to performing the reaction in OSM (Karboune *et al*., 2008). The present work was aimed at the enzymatic synthesis of potential phenolic lipids by the transesterification of flaxseed oil and DHPA in SFM. The specific objectives were to investigate the effects of water activity, different enzyme concentrations at various agitation speeds and Silica gel addition on the enzymatic synthesis of phenolic lipids. The molecular structures of the synthesized phenolic lipids were characterized and their free radical scavenging activity was also determined.

4.3. Materials and Methods

4.3.1. Materials

Immobilized lipase from *Candida antarctica* (Novozym 435) with an activity of 10,000 propyl laurate units, PLU, per g solid enzyme, was obtained from Novozymes Nordisk A/S (Bagsværd, Denmark). 3,4-dihydroxyphenyl acetic acid, Silica gel (60-200 mesh), salt hydrates pairs, including lithium chloride 1/0, sodium carbonate 1/0, barium hydroxide 8/1, sodium pyrophosphate 10/0 (where the salt hydrate pairs are referred to by a shorthand notation giving the number of water molecules), sodium methoxide and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) were purchased from Sigma Chemical Co. (St-Louis, MO). Flaxseed oil was a gift from Arista Industries, Inc. (Wilton, CT). Gas liquid chromatography (GLC) standards were purchased from Nu-check Prep (Elysian, MN). Organic solvents of high-performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific (Fair Lawn, N.J.).

4.3.2. Transesterification Reaction in Solvent-Free Medium

Lipase-catalyzed transesterification of flaxseed oil with 3,4-dihydroxyphenyl acetic acid (DHPA) was carried out, according to the modification of the method of Sabally *et al*. (2006b) developed in our laboratory (Chapter III). Prior to each enzymatic reaction, a stock solution of the phenolic acid (143 mM) in 2-butanone was freshly prepared. Aliquot of the phenolic acid stock solution was mixed with flaxseed oil to acquire a final concentration of 10 mM, where 2-butanone was 7% of the total reaction volume. The enzymatic reaction was initiated by the addition of 50 mg of solid enzyme (500 PLU)/mL. The reaction mixture was incubated at 55°C, with continuous shaking at 150 rpm, in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The transesterification reactions were run in duplicate in tandem with control trials that contain all components except the enzyme. The enzymatic reaction was monitored at selected time intervals over the course of 9 days. The recovered samples were flushed with a gentle stream of nitrogen and stored at – 80ºC for further analysis.

4.3.3. Effect of Water Activity

Salt hydrate pairs were used to control water activity in the reaction mixture, including lithium chloride 1/0 (a_w 0.05), sodium carbonate 1/0 (a_w 0.38), barium hydroxide 8/1 (a_w 0.47) and sodium pyrophosphate 10/0 (a_w 0.67) at a concentration of 0.1 g/mL reaction mixture of each salt

(Halling, 1992). The water activity (a_w) was measured by a Novosina AW SPRINT TH-500 system (Axair Ltd., Pfaffikon, Switzerland) using humidity reference points at 25°C.

4.3.4. Characterization of End Products

4.3.4.1. HPLC Analysis of the Reaction Components

HPLC analysis of reaction components was carried out according to the method developed in our laboratory (Sabally *et al.,* 2006b). The separation was performed on an Agilent Zobrax SB-C18 reversed-phase column (250x4.6 mm, 5 µm), using a Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). A volume of 20 µL sample was recovered and solubilized in 150 µL isopropanol. A volume of 20 µL of the diluted sample was subjected to HPLC analysis. The elution of the injected sample was carried out by a gradient solvent system, using acetonitrile/methanol mixture (7:5, v/v) as solvent (A) and isopropanol as solvent (B). The elution was initiated by an isocratic flow of 100% of solvent A for 10 min, followed by a 10 min linear gradient to 40 and 60% of solvent A and B, respectively, then to 100% of solvent B for 10 min period. The elution was maintained for an additional period of 5 min before reverting to the initial conditions (100% solvent A), followed with an equilibration period of 10 min for the next sample. The flow rate was at 1 mL/min and the detection was performed at 280 nm for monitoring the phenolic lipids products.

The maximal bioconversion yield of phenolic lipids was calculated as the total peak area of phenolic lipids, detected at 280 nm, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100. The enzymatic activity was calculated from the slope of the linear portion of the plot of bioconversion yield versus the reaction time and it was expressed as nmol of PLs per g solid enzyme per min of reaction.

4.3.4.2. Mass Spectrometry Analysis of End Products

The characterization of reaction components of the lipase-catalyzed transesterification reaction was also performed by HPLC interfaced to an atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The APCI-MS system (ThermoFinnigan, San Jose, CA) was equipped with the Zorbax SB-C18 reversed-phase column as well as a Surveyor liquid chromatography

pump, an autosampler and Xcalibur® system control software (version 1.3) for data acquisition and processing. The mass spectrometer was operated in positive ion mode with a collision energy source of 15 V. The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

4.3.5. Effect of Lipase Concentration and Agitation Speed

The effect of the amount of the biocatalyst, Novozym 435, was investigated by varying its concentrations from 40 to 70 mg of solid enzyme (400 to 700 PLU)/mL, the enzymatic reactions were carried out at different agitation speeds (0, 100, 150 and 200) rpm and monitored at day five.

4.3.6. Effect of Silica Gel

Over a 9 day time period of reaction, the transesterification reaction was conducted in the presence of Silica gel (60-200 mesh); at a concentration of 1.2 and 2.5 mg/mL of the reaction mixture based on Karboune *et al*. (2005) method.

4.3.7. Determination of Relative Fatty Acid Composition

Fatty acids composition of the flaxseed oil and of the phenolic mono- and diacylglycerols, recovered by preparative HPLC analysis, was investigated using GLC. Prior to GLC analysis, 5 mg of each recovered phenolic mono- and diacylglycerols fractions were diluted in 0.6 mL of hexane and methylated according to the method of Rocha-Uribe and Hernandez (2004), using 60 μL of 2 M sodium methoxide in 20% methanol. The mixture was incubated in reciprocal shaking water-bath (Model 25, Precision Scientific, Chicago, IL) at 65ºC. After 20 min of incubation, 10% sulfuric acid solution prepared in methanol was added followed by its incubation in a water-bath at 85ºC for 30 min. The methylated fatty acids were recovered with hexane. The upper layer was collected and recovered for GLC analysis. The analysis of fatty acid methyl esters was carried out using Agilent 6890 chromatography system (Agilent Technologies, Wilmington, DE), equipped with a FID detector, a split injector and Supelco Omega Wax 24152 fused capillary column (30 m×0.32 mm I.D.×0.25 μm film thickness). The injector and detector temperatures were set at 250°C. The column temperature was maintained at 150°C for 3 min and then increased to 200°C (1°C/min), which was held for 15 min followed by an additional increase to 250°C over a period of 5 min. The helium was used as the carrier gas, with a flow

rate of 1 mL/min and a pressure of 9.8 psi, whereas the air and hydrogen were 400 and 40 mL/min, respectively. Relative fatty acid percentages were compared by paired t-tests at $P < 0.05$.

4.3.8. Determination of the Free Radical Scavenging Activity of Phenolic Lipids

The free radical scavenging activity of phenolic lipids was determined according to a modification of Silva *et al.* (2000) method, using $DPPH^{\bullet}$ as the stable free radical compound. Prior to the analysis, a stock solution of $DPPH^{\bullet}$ (0.14 mM) was freshly prepared in absolute ethanol. The transesterified phenolic acids were recovered upon hydrolysis of phenolic lipids with 4 M NaOH for 4 h (Sosulski *et al*., 1982) and their concentrations were quantified, using Folin-Ciocalteu method (Singleton, 1965).

The investigated phenolic lipids as well as references were solubilized in the ethanolic solution of $DPPH^{\bullet}$ to yield a final concentration of (10, 20, 30, 50, 75, 100 and 150 µM). The decrease in $DPPH^{\bullet}$ concentration was followed spectrophotometrically with Beckman spectrophotometer (Model 650, Inc., Fullerton, CA) at 517 nm over a period of 80 min, using a blank assay without $DPPH^{\bullet}$. In addition, a control reaction containing only $DPPH^{\bullet}$ free radical was carried out in tandem with the scavenging reaction trial. The radical scavenging activity of the investigated compounds were compared on the basis of IC_{50}, which represents the concentration needed to reduce 50% of the initial amount of $DPPH^{\bullet}$ expressed in µM. Scavenging activity (SA) % versus sample concentration (SC) curve was plotted and the IC_{50} was calculated by non linear regression using SigmaPlot Program (version 11, Jandel Scientific, Germany). Data were found to fit the curve equation: $[SA\ \%] = A.e^{\,b[SC]}$. All trials were performed in triplicate and the percentage of residual $DPPH^{\bullet}$ was calculated using absorbance of the reaction mixture at 517 nm (A_{max}), at a defined time, divided by the initial absorbance of $DPPH^{\bullet}$ control (A_i), multiplied by 100. The scavenging $DPPH^{\bullet}$ percentage was determined by subtracting the residual $DPPH^{\bullet}$ percentage from 100, multiplied by 100, where, A_{max} is the maximal absorbance of the phenolic products mixture after it reached the steady state, and A_i is the initial absorbance of the $DPPH^{\bullet}$ control trial.

4.3.9. Statistical Analysis

All experiments data were analyzed as completely randomized design using PROC ANOVA of the statistical analysis system (SAS, 2009). Effect of lipase concentration and agitation speed experiment data were statistically analyzed as factorial design. Multiple comparisons of mean values were done by Tukey's honest significance test at $P < 0.05$.

4.4. Results and Discussion

4.4.1. Effect of Water Activity

The enzymatic synthesis of phenolic lipids by the transesterification of flaxseed oil with DHPA in SFM was investigated, using Novozym 435 from *Candida antarctica* as the biocatalyst (Fig. 9). The water activity (a_w) of the reaction medium is an important parameter, since it affects both the enzymatic activity and the reaction equilibrium (Valivety *et al.,* 1992). The effect of a_w on the reaction time course for lipase-catalyzed transesterification of flaxseed oil with DHPA in SFM is shown in Figure 14. At a_w values of 0.05, 0.47 and 0.67, the total bioconversion yield increased to a maximum value of 34, 31 and 28%, respectively, after 5 days of reaction and then remained relatively constant over an additional 4 days of reaction. At a_w of 0.18 the bioconversion yield increased rapidly within the first 3 days of reaction to 52%, and to a lower extent thereafter up to a maximum of 63% after an additional 4 days. Adjusting the a_w to 0.38 resulted in a significant increase ($P < 0.05$) in the bioconversion yield up to 77% after 5 days of the reaction. At a_w of 0.18 and 0.38, the bioconversion yield decreased to 41 and 67%, respectively, after an additional 2 days, this decrease may be due to a shift in the thermodynamic equilibrium of the reaction toward hydrolysis and/or to the acyl migration, as a result of increase in the concentration of free fatty acids (Karboune *et al.,* 2005).

The overall results (Fig. 14) indicate that the maximum bioconversion yield increased significantly ($P < 0.05$) from 34 to 63% when the a_w was increased from 0.05 to 0.18; further increase of a_w to 0.38 resulted in a significant increase ($P < 0.05$) of bioconversion yield up to 77%. These results may be attributed to the low flexibility of enzyme at low water environment, which may have reduced its ability to undergo effective conformational changes required to form enzyme-substrate complexes (Gomez-Puyou and Gomez-Puyou, 1998). In addition, a thin monolayer of bound water is needed to maintain the enzyme's three dimensional structure needed to retain its catalytic activity (Halling, 2002). However, further increase of a_w from 0.38

to 0.47 and 0.67 resulted in a significant decrease ($P < 0.05$) in the maximum bioconversion yield to 31 and 28%, respectively; this decrease may be due to a shift in the reaction thermodynamic equilibrium towards hydrolysis (Karboune *et al*., 2005). In addition, Graille (1999) reported that lipase-catalyzed reactions in SFM require a minimal hydration to maintain its activity toward ester synthesis. Similarly, Karra-Chaabouni *et al.* (2002) reported that a high bioconversion yield (75%) of geranyl butyrate was obtained at low initial a_w of 0.2 to 0.5 using an esterase from *Mucor miehei* in SFM; while a lower bioconversion yield (13%) was obtained with initial a_w higher than 0.5. In contrast, Lue *et al*. (2005) reported that low initial a_w value of 0.05 was found to be the most suitable one for the esterification of cinnamic acid with oleyl alcohol in OSM, and it resulted in the highest enzymatic activity (193 nmol of PLs/g solid enzyme/min) and a bioconversion yield of 75%.

4.4.2. Structural Characterization of Phenolic Lipids

The HPLC elution profiles of the reaction components of the lipase-catalyzed transesterification reaction of flaxseed oil, with DHPA at a_w of 0.38, was monitored at 280 nm. Peak # 1 (Figs. 15A and 15B), was characterized as DHPA. Six predominant peaks (# 2, 3, 4, 5, 6 and 7) were characterized as the main phenolic lipids of the transesterification reaction, since they have shown a UV-spectral scanning profile comparable to that of its phenolic acid component (peak # 1). TAGs of flaxseed oil were identified as peaks # 8 to 13 in the elution profiles of the reaction components (Figs. 15A and 15B).

In order to characterize the molecular structure of the synthesized phenolic lipids, further analysis by HPLC/APCI-MS spectrometry of the eluting peaks was conducted. The phenolic group substitution on the glycerol backbone of the phenolic lipids is shown for simplicity, as the regioselectivity of the reaction is unknown (Karboune *et al.,* 2008). The fragmentation patterns of the main phenolic lipids products are shown in Figure 16. The fragmentation of peak #2 (Fig. 15B) produced a fragment ion at *m/z* 335.3 representing monolinolenin as well as abundant molecular ions at 485.3 $[M+ H–H_2O]^+$ and m/z 503.3 $[M+H]^+$, which are characteristics of monolinolenyl 3,4-dihydroxyphenyl acetate (Fig. 16A). While the fragmentation of peak #3 (Fig. 15B) produced a fragment ion at *m/z* 754.0 $[M+ H–H_2O]^+$ which is characteristics of dioleyl 3,4-dihydroxyphenyl acetate (Fig. 16B).

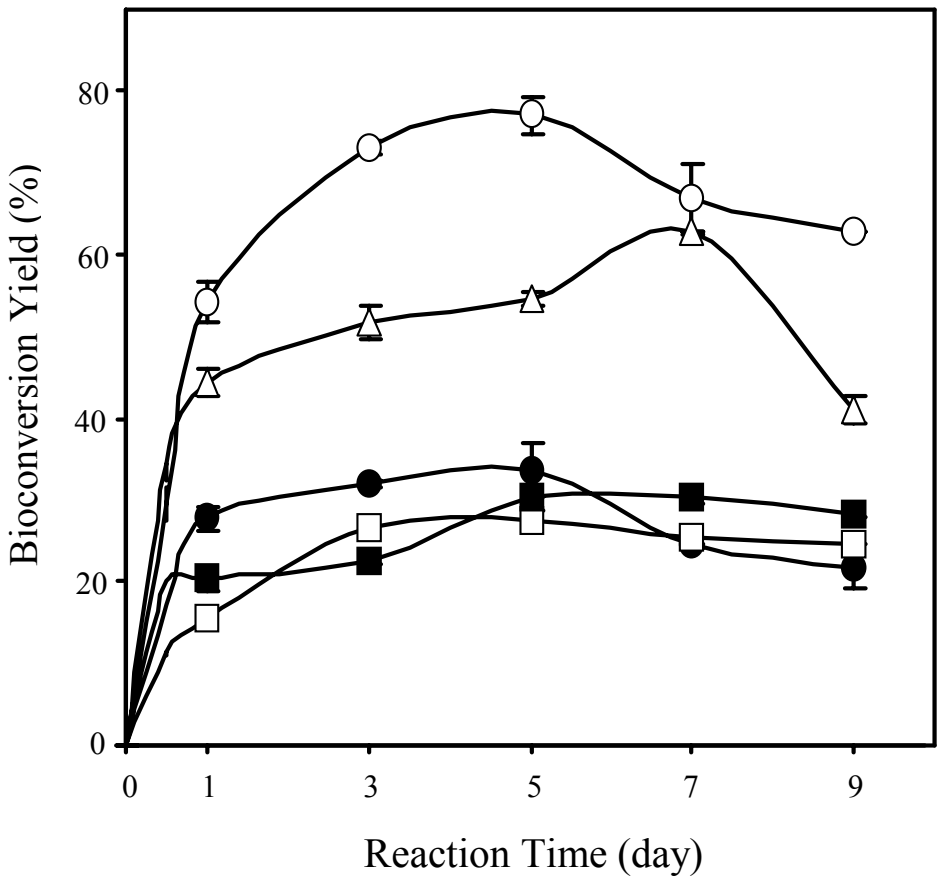


Figure 14. Effect of different water activity on the bioconversion yield of phenolic lipids, using salt hydrates, including lithium chloride with a_w 0.05 (●), sodium carbonate with a_w 0.38 (o), barium hydroxide with a_w 0.47 (□), sodium pyrophosphate with a_w 0.67 (■) and control trial without salt at a_w 0.18 (▲), STD ≤ 4.1.

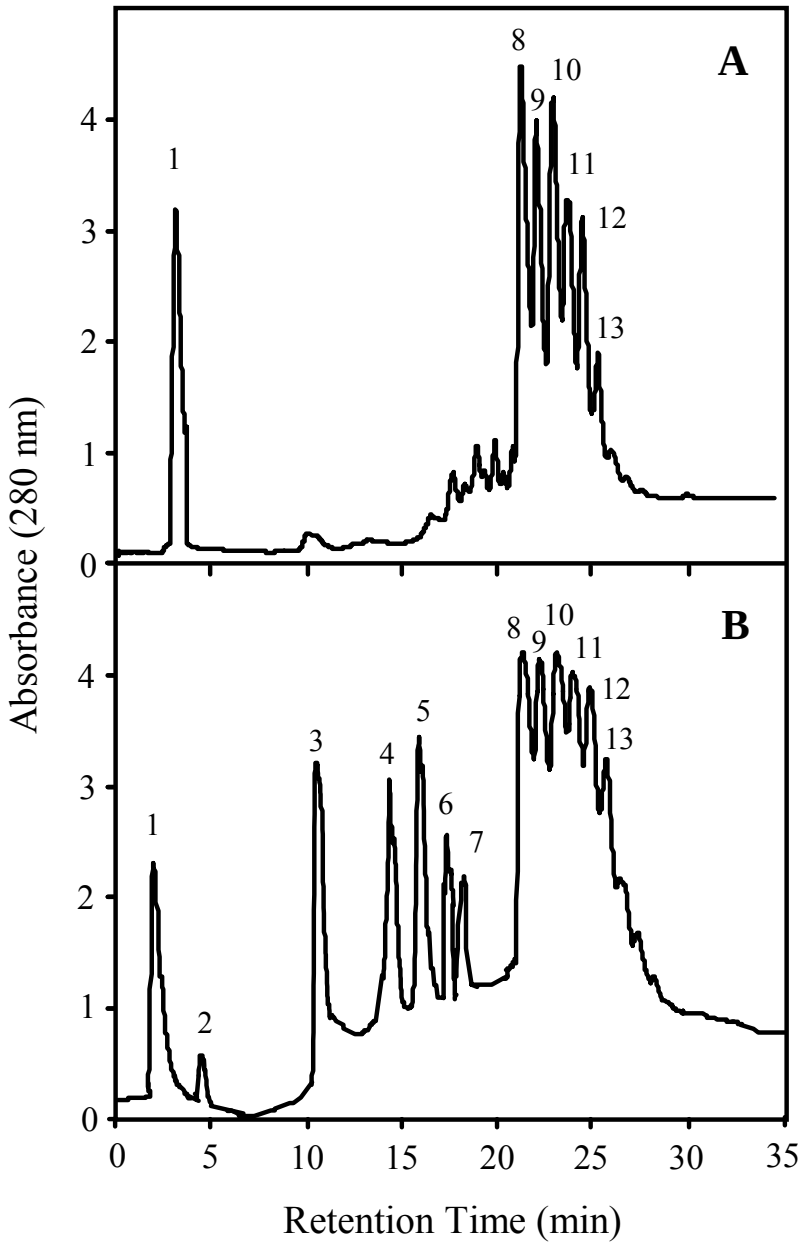


Figure 15. HPLC chromatograms of the initial reaction mixture of dihydroxyphenyl acetic acid (DHPA) with flaxseed oil (A), the enzymatic transesterification reaction mixture (B) monitored at 280 nm. Peak numbers were identified as follows: DHPA #1, phenolic monoacylglycerols #2, phenolic diacylglycerols #3, 4, 5, 6, 7 and flaxseed oil #8, 9, 10, 11, 12 and 13.

The results also indicate that the fragmentation pattern of peak #4 (Fig. 15B) possess a major molecular ion $[M]^+$ at *m/z* 763.5 corresponding to dilinolenyl 3,4-dihydroxyphenyl acetates (Fig. 16C). Peak #5 (Fig. 15B) was characterized as linoleyl linolenyl 3,4-dihydroxyphenyl acetate (Fig. 16D), with an abundant molecular ion $[M]^+$ at *m/z* 765.6 and different fragment ions at *m/z* 335.4, 337.5 and 615.0, corresponding to monolinolenin $[M+ H–H_2O]^+$, monolinolein $[M+ H–H_2O]^+$ and linoleyl linolenyl glycerol $[M+H]^+$, respectively. While peak #6 (Fig. 15B) fragmentation resulted in a characteristic pattern of oleyl linolenyl 3,4-dihydroxyphenyl acetate (Fig. 16E), with an abundant molecular ion at *m/z* of 767 $[M]^+$ and fragment ions at 335.3, 339.4 and 617.6, corresponding to monolinolenin $[M+ H–H_2O]^+$, monoolein $[M+ H–H_2O]^+$ and oleyl linolenyl glycerol $[M+H]^+$, respectively. In addition, The fragmentation of peak #7 (Fig. 15B) resulted in a characteristic pattern of oleyl linoleyl 3,4-dihydroxyphenyl acetate (Fig. 16F), with an abundant molecular ion at *m/z* of 769.0 $[M]^+$ and fragment ions at 337.3, 339.4 and 619.6, corresponding to monolinolein $[M+ H–H_2O]^+$, monoolein $[M+ H–H_2O]^+$ and oleyl linoleyl glycerol $[M+H]^+$, respectively.

The loss of H_2O, from a monoacylglycerol during HPLC/APCI-MS analysis, has been previously reported (Sabally *et al.,* 2006b; Safari *et al.,* 2006; Karboune *et al*., 2008; Karam *et al.*, 2009). The HPLC/APCI-MS analysis confirmed the formation of various phenolic mono- and diacylglycerols by lipase-catalyzed transesterification of flaxseed oil with DHPA in SFM. Likewise, Sabally *et al.* (2006b) and Karboune *et al.* (2008) have characterized 6 dihydrocaffeoylated lipids as well as 6 dihydroxyphenyl acetoylated lipids (monolinolenyl, monolinoleyl, dilinolenyl, dilinoleyl, linoleyl linolenyl and oleyl linolenyl 3,4-dihydroxyphenyl acetates) as the end products of lipase-catalyzed transesterification of flaxseed oil in OSM, with DHCA and DHPA, respectively, as substrates.

4.4.3. Effect of Lipase Concentration and Agitation Speed

In order to maximize the bioconversion yield and reduce the reaction time, the lipase-catalyzed transesterification reaction was carried out using a wide range of enzyme concentrations, varying from 40 to 70 mg of solid Novozym 435 (400-700 PLU)/mL at agitation speed range (0-200) rpm in SFM. The effect of the enzyme concentration at different agitation speeds on both the enzymatic activity and the bioconversion yield of phenolic lipids were investigated. Figure 17 show that the enzymatic activity decreased significantly ($P < 0.05$) with increasing enzyme

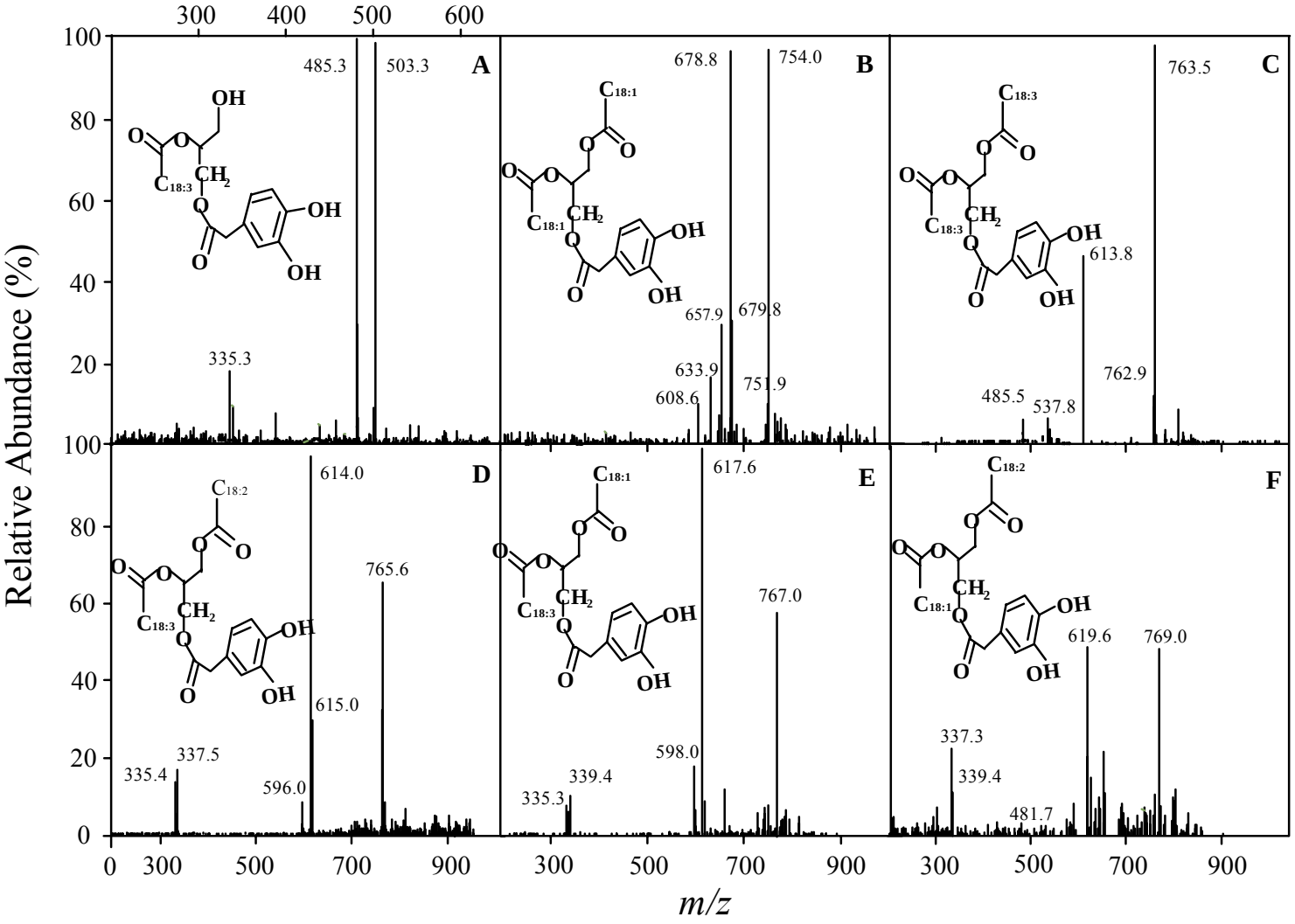


Figure 16. Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) analysis of 3,4-dihydroxyphenyl acetoylated monoacylglycerol (A), and -diacylglycerols (B, C, D, E and F).

concentration from 40 to 70 mg/mL at all investigated agitation speeds (0 to 200 rpm). However, at agitation speed 150 rpm, the highest enzymatic activity (178 nmol of PLs/g solid enzyme/min) was obtained with enzyme concentration 40 mg/mL, which was decreased to 139 nmol of PLs/g solid enzyme/min when the enzyme concentration was increased to 50 mg/mL. While increasing the enzyme concentration to 70 mg/mL resulted in a further significant decrease ($P < 0.05$) in the enzymatic activity to 100 nmol of PLs/g solid enzyme/min (Fig. 17). These results may be due to the mass transfer limitation at a high enzyme concentration, which could have affected the diffusion of the substrate to the enzyme active site and hence its availability for the reaction (Palmer, 1995). The decrease in the enzyme activity at high enzyme concentration may be due to the steric hindrance of its active site (Hadzir *et al.*, 2001).

The presence of high protein-support interactions as a result of an excess of the enzyme could have altered its active conformation and hence its catalytic efficiency (Yadav and Lathi, 2003). In addition, the limited increase in the enzyme activity can be explained by the increase in the concentrations of glycerol and or/free fatty acid as by-products as a result of the hydrolysis of TAGs. Indeed, the accumulation of glycerol molecules on the enzyme micro-environment may lead to the formation of the hydrophilic hindrance layer, limiting the substrate diffusion and hence the bioconversion yield of phenolic lipids (Dossat *et al.,* 1999). Similarly, Karboune *et al.* (2005) reported that the formation of a hydrophilic hindrance layer of glycerol around the enzyme molecule could be a valid explanation for the limited increase in the bioconversion yield of cinnamoylated lipids. On the other hand, increasing the enzyme concentration from 40 to 70 mg/mL of solid Novozym 435 (400-700 PLU) at agitation speed 150 rpm resulted in a bioconversion yield in the range of 73 to 79 (data not shown). These results indicate that varying the enzyme concentration within this range had no significant effect on the maximum bioconversion yield. Similarly, Awang *et al.* (2004) reported that an excess of Novozym 435 (> 0.2 g) did not increase the esterification yield of oleic acid with oleyl alcohol. In addition, Karam *et al*. (2009) reported that the highest bioconversion yield (83%) of dihydroxyphenyl acetoylated lipids was obtained with 50 mg of solid enzyme (500 PLU), using fish liver oil and DHPA as substrates in OSM, while using 60 mg of solid enzyme the bioconversion yield was decreased to 60%. Thus the increase in the enzyme concentration without the presence of adequate amount of substrate results in a decrease in the esterification yield (Krishna, 2002).

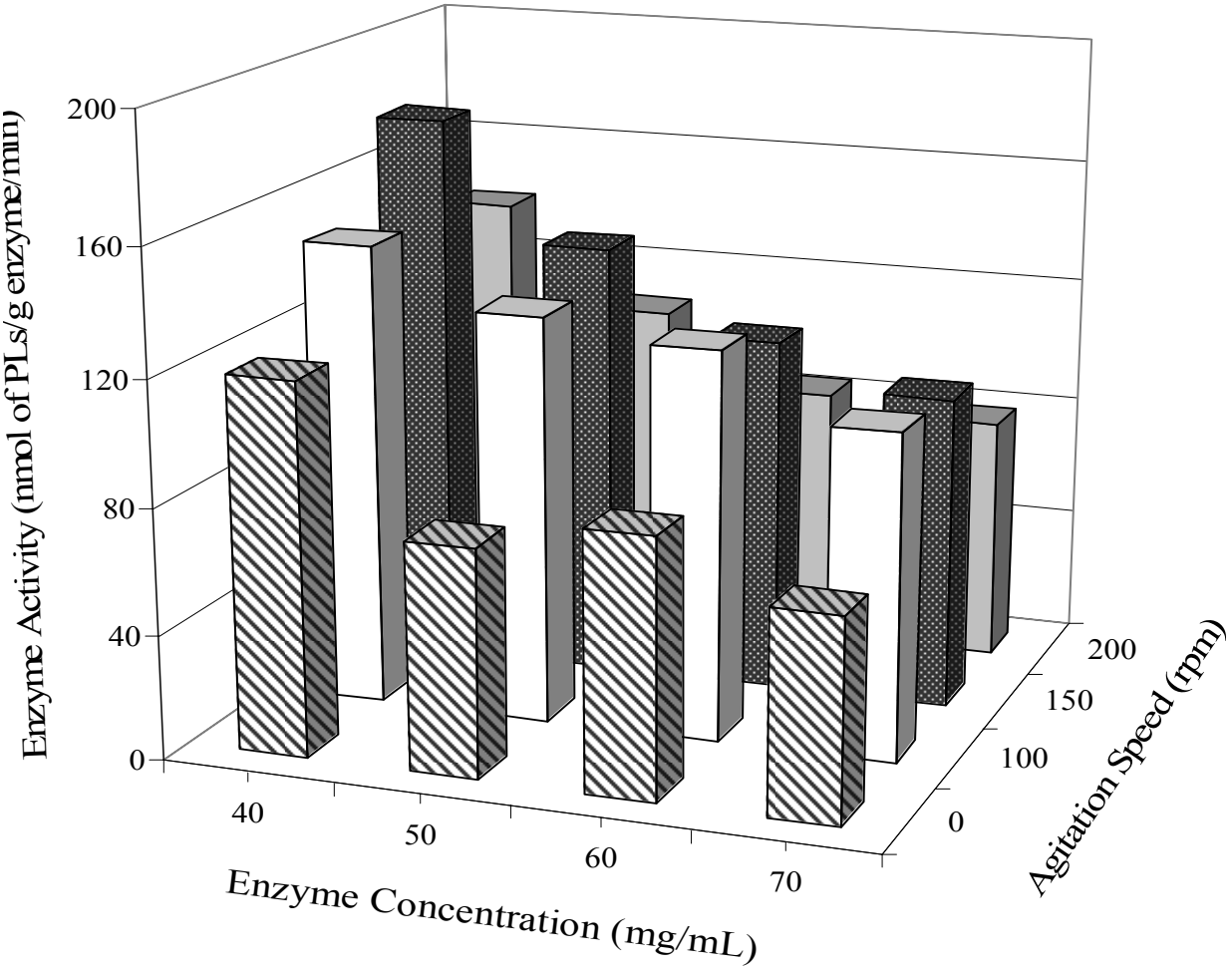


Figure 17. Effect of Novozym-435 lipase concentration and agitation speed on the enzymatic activity following a series of 5-days transesterification reaction using 3,4-dihydroxyphenyl acetic acid and flaxseed oil as substrates in solvent-free medium.

Likewise, Senanayake and Shahidi (2002) indicated that the extent of docosahexaenoic acid incorporation into borage oil was increased by increasing the amount of the enzyme in the mixture; however a significant increase was not obtained when the amount of enzyme was greater than 100 units.

In a heterogeneous enzymatic system, it is important to ensure that the rate of substrate diffusion does not limit the rate of the enzymatic synthesis reaction (Barros *et al*., 1998). Using 40 mg of solid enzyme (400 PLU)/mL, a significant increase ($P < 0.05$) in the enzymatic activity from 119 to 178 nmol of PLs/g solid enzyme/min was obtained when the agitation speed of the system was increased from 0 to 150 rpm, and then decreased to 140 nmol of PLs/g solid enzyme/min when the agitation speed was further increased to 200 rpm (Fig. 17). Similarly, Hadzir *et al*. (2001) reported that an agitation speed of 150 rpm was selected for the enzymatic alcoholysis of triolein by Novozym 435. The increase in the agitation speed may decrease the boundary liquid layer surrounding the porous support, resulting in lower diffusional limitations (Barros *et al*., 1998). Similar results were obtained by Lue *et al.* (2005) for the esterification of cinnamic acid and oleyl alcohol in OSM. On the basis of these findings, 40 mg of solid Novozym 435 (400 PLU)/mL at agitation speed 150 rpm was found to be the most effective biocatalyst amount and agitation speed for the incorporation of DHPA into flaxseed oil, and consequently used for further investigation.

4.4.4. Effect of Silica Gel Addition

In order to limit the formation of the glycerol hydrophilic hindrance layer in the enzyme micro-environment, Silica gel was added to the reaction mixture. The enzymatic synthesis of phenolic lipids was investigated upon the addition of acidic and alkaline Silica gel at a concentration of 1.2 and 2.5 mg/mL (Fig. 18). The results obtained indicate that both acidic and alkaline Silica gel has similar time course trend and effect on the bioconversion yield. The bioconversion yield remained relatively constant (70%) upon the addition of alkaline Silica gel at concentration 1.2 mg/mL, while the enzymatic activity was decreased from 174 to 109 nmol of PLs/g solid enzyme/min. In addition, upon increasing the concentration of Silica gel from 1.2 to 2.5 mg/mL the bioconversion yield and the enzymatic activity were decreased significantly ($P < 0.05$) from 73 to 54% and from 174 to 89 nmol of PLs/g solid enzyme/min, respectively, after 7 days of reaction. Nevertheless, in the absence of Silica gel, a higher bioconversion yield and an

enzymatic activity of 73% and 174 nmol of PLs/g solid enzyme/min, respectively, were obtained. These findings could be explained by the dehydrating effect of Silica gel on the enzyme, which may have resulted in its partial inactivation (Stevenson *et al.,* 1994). In addition, a possible partition of glycerol between the Silica gel and the enzyme support may have also occurred (Dossat *et al.,* 1999). On the contrary, Karboune *et al.* (2005) reported an increase in the maximum bioconversion yield of cinnamoylated lipids, from 42 to 55% upon the addition of 2.2 mg Silica gel/mL to the reaction mixture; however, the bioconversion yield decreased by 26% upon an increase in the Silica gel concentration to 4.4 mg/mL. On the basis of the experimental findings (Fig. 18), it was concluded that the transesterification reaction of DHPA and flaxseed oil was shown to be more effective in the absence of Silica gel.

4.4.5. Determination of Relative Fatty Acid Composition

In order to evaluate the changes in the relative fatty acid composition of the flaxseed oil TAGs, following its transesterification with DHPA, the original oil and its phenolic lipids fractions were subjected to GLC analysis. The results (Table 7) show that the predominant fatty acids in flaxseed oil were oleic ($C_{18:1}$ *n*-9), linoleic ($C_{18:2}$ *n*-6) and linolenic ($C_{18:3}$ *n*-3) acids, with 18.0, 16.5 and 57.7% of the total fatty acids, respectively. However, the relative proportions of these fatty acids in the synthesized phenolic lipids were different, indicating hence a modification in the profile of fatty acids of flaxseed oil upon its transesterification with DHPA. The proportion of linolenic acid was increased significantly ($P < 0.05$) from 57% in the flaxseed oil to 75 and 64% in the phenolic mono- and diacylglycerols, respectively, whereas that of oleic acid was decreased from 18% to 8 and 14%, respectively. The results (Table 7) also show a significant decrease ($P < 0.05$) in the proportions of palmitic and stearic acids in the synthesized phenolic lipids as compared to that in the flaxseed oil. The overall results suggest a higher specificity of lipase for the exchange of the acyl group of stearic, palmitic and oleic acid, with that of phenolic acid as compared to that of linoleic and linolenic acids. Similarly, Sabally *et al.* (2006b) and Karboune *et al.* (2008) have also reported an increase in the relative proportion of linolenic acid, as well as a decrease in those of oleic and linoleic acids upon the incorporation of DHCA and selected phenolic acids into flaxseed oil in OSM, respectively.

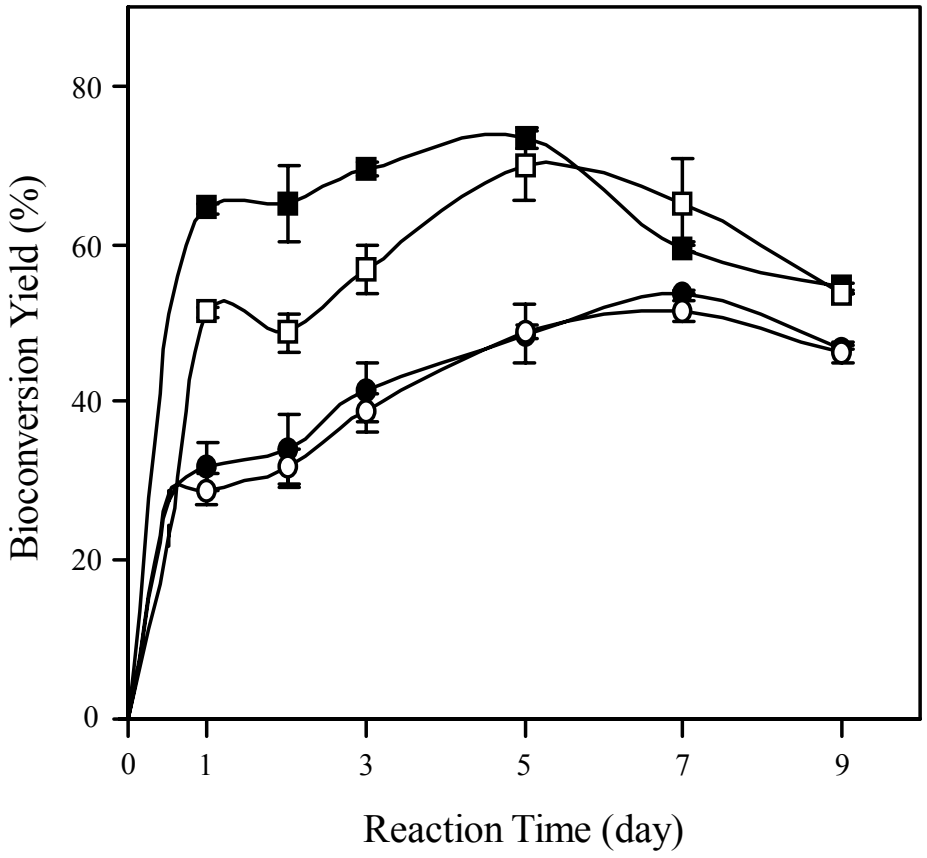


Figure 18. Time course for the transesterification reaction of flaxseed oil with 3,4-dihydroxyphenyl acetic acid , in the presence of Silica gel [(acidic Silica gel 2.5 mg (o), alkaline Silica gel 2.5 mg (●), alkaline Silica gel 1.2 mg (□)] and in the absence of Silica gel, control trial (■), STD ≤ 4.8.

Table 7. Relative fatty acid composition (%) of flaxseed oil before and after modifications by transesterification with 3,4-dihydroxyphenyl acetic acid in solvent-free medium.

	Relative fatty acid (%)[a]		
Fatty acid	Flaxseed oil	Phenolic monoacylglycerols	Phenolic diacylglycerols
$C_{16:0}$	4.75 (7.1)[b]	0.15 (3.5)[b]	0.71 (1.4)[b]
$C_{18:0}$	3.50 (12.0)[b]	0.46 (12.0)[b]	2.74 (6.2)[b]
$C_{18:1}$ *n-9*	18.00 (7.9)[b]	7.95 (9.5)[b]	14.65 (10.0)[b]
$C_{18:2}$ *n-6*	16.50 (4.3)[b]	16.20 (12.6)[b]	17.43 (6.7)[b]
$C_{18:3}$ *n-3*	57.70 (9.2)[b]	75.24 (3.6)[b]	64.47 (1.7)[b]

[a]Relative fatty acid (%) was calculated as the peak area of each fatty acid divided by the total peak area of all fatty acids, multiplied by 100.

[b]Data are average of duplicate samples and relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

4.4.6. Determination of the Radical Scavenging Activity of Phenolic Lipids

Among naturally occurring phenolic compounds, phenolic acids and their derivatives are recognized as natural potent antioxidants, exhibiting radical scavenging activity (Silva *et al.,* 2000). It has been noted that the radical scavenging activity of phenolic acids and their derivatives is dependent on the number of hydroxyl and methoxyl groups on the benzene ring (Cuppett *et al.,* 1997). The radical scavenging activity of DHPA and its phenolic lipid esters toward the stable free radical $DPPH^{\bullet}$ was evaluated and compared with that of α-tocopherol.

The overall results (Table 8) showed that the steady state of the reaction between $DPPH^{\bullet}$ and DHPA as well as α-tocopherol was reached in 7.5 to 36 min and 1.5 to 24 min, respectively, for all investigated concentrations (Table 8), while for the tested phenolic lipids it was reached in 32 to 64 min. In addition, the radical scavenging activity of the tested compounds were compared on the basis of IC_{50}, which represents the concentration needed to reduce 50% of the initial amount of $DPPH^{\bullet}$, expressed in μM. The experimental findings indicated that 11.1 μM of DHPA was required to reduce 50% of the initial amount of $DPPH^{\bullet}$, whereas 41.2 and 34.4 μM of 3,4-dihydroxyphenyl acetoylated lipids and α-tocopherol were needed, respectively, to attain the same decrease. These results suggest that the acylation of DHPA reduced its radical scavenging activity by 3.7-fold. The attachment of a mono- or diacyglycerol moiety to the side chain of DHPA might have reduced or restricted the rotation degree of its phenyl moiety along the side chain, leading to a reduction in its ability to scavenge the free radicals (Sabally *et al*., 2007; Karam *et al*., 2009). Likewise, Karboune *et al*. (2008) reported that DHPA showed higher radical scavenging ability (82%), whereas 3,4-dihydroxyphenyl acetoylated lipids and α-tocopherol scavenged 29 and 35% of $DPPH^{\bullet}$, respectively. Overall, the 3,4-dihydroxyphenyl acetoylated lipids demonstrated radical scavenging ability compared to that of α-tocopherol with no significant difference ($P > 0.05$), indicating their potential as antioxidants.

Table 8. Radical scavenging effect of phenolic lipids and its corresponding phenolic acid.

Phenolic compound	Time to reach steady state (min)[a]	IC_{50}[b]
3,4-Dihydroxyphenyl acetic acid	7.5 – 36.0	11.1 (8.5)[c]
3,4-Dihydroxyphenyl acetoylated lipids	32.0 – 64.0	41.2 (3.4)[c]
α-Tocopherol	1.5 – 24.0	34.4 (3.6)[c]

[a]Determination of the steady state at concentrations range (10-150 μM).

[b]IC_{50} represents the concentration of phenolic compounds in μM needed to reduce 50% of the initial amount of $DPPH^{\bullet}$.

[c]Data are average of three measurements and the relative standard deviations was calculated from the standard deviation of triplicate samples divided by their mean, multiplied by 100.

4.5. Conclusion

A promising work towards a clean bioconversion was conducted in our lab, where the highest bioconversion yield of phenolic lipids was obtained from the transesterification of DHPA with flaxseed oil, in SFM at a_w of 0.38. The reaction led to a significant increase in the relative proportion of linolenic acid ($C_{18:3}$ ω-3) in the synthesized phenolic lipids as compared to that in the unmodified flaxseed oil. In addition, the synthesized phenolic lipids demonstrated radical scavenging activity compared to that of α-tocopherol indicating their potential as antioxidants.

CHAPTER V

STATEMENT OF CHAPTER V LINKAGE

In order to expand the use of solvent-free medium, structuring of fish liver oil with selected phenolic acids for the enzymatic synthesis of novel phenolic lipids was investigated. Research work conducted previously in Chapters III and IV showed the validity of the biotechnological approach for clean enzymatic synthesis of phenolic lipids from flaxseed oil, with high productivity, enhanced solubility and anti-oxidative properties. The presented work in this Chapter investigated the effects of phenolic acid structure and surfactants on the bioconversion yield of phenolic lipids from fish liver oil. The molecular structures of the synthesized phenolic lipids and their free radical scavenging activity were characterized. In addition, the oxidative stability of the novel phenolic lipids was determined to investigate the effect of phenolic acid incorporation into the oil, and compared to the unmodified fish liver oil.

CHAPTER V

LIPASE-CATALYZED SYNTHESIS OF PHENOLIC LIPIDS IN SOLVENT-FREE MEDIUM USING FISH LIVER OIL AND SELECTED PHENOLIC ACIDS

5.1. Abstract

Phenolic lipids (PLs) were obtained by lipase-catalyzed transesterification of fish liver oil with selected phenolic acids in solvent-free medium (SFM), including hydroxylated and/or methoxylated derivatives of cinnamic and phenyl acetic acids. A bioconversion yield of 61% was obtained for the transesterification of fish liver oil with dihydrocaffeic acid (DHCA). The enzymatic activity was increased from 177 to 240 nmol of PLs/g solid enzyme/min upon the addition of the non-ionic surfactant Span 65. The results showed a significant increase in the relative proportions of the two highly desirable essential fatty acids eicosapentaenoic acid (EPA, $C_{20:5}$ *n*-3) and docosahexaenoic acid (DHA, $C_{22:6}$ *n*-3). EPA was increased from 11.5% in the fish liver oil to 21.2, 20.7, 20.8, 20.1 and 19.8%, in dihydrocaffeoylated, 3,4-dihydroxyphenyl acetoylated, caffeoylated, feruloylated and sinapoylated lipids, respectively, while, DHA increased from 12.0% in the fish liver oil to 21.4, 19.4, 27.5, 22.1 and 22.0%, respectively. The synthesized phenolic lipids demonstrated radical scavenging activity, expressed as IC_{50} from 1.6 to 3.3-fold higher than that of its corresponding phenolic acid, but compared to that of α-tocopherol. In addition, the synthesized phenolic lipids demonstrated higher oxidative stability as compared to the unmodified fish liver oil.

5.2. Introduction

Because of their wide range of biological and cellular functions, polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, $C_{20:5}$ *n*-3) and docosahexaenoic acid (DHA, $C_{22:6}$ *n*-3), are effective against many diseases, including cardiovascular and autoimmune diseases, rheumatoid arthritis and cancers (Larsson *et al*., 2004; Riediger *et al*., 2009). In addition, DHA being the major PUFA found in human brain membranes and retina plays a major role in their proper functioning and in the development of the central nervous system of infants (Nettleton, 1993).

Fish oils, including that of menhaden, sardine, anchovy, herring and cod liver are the main dietary source of EPA and DHA with a total amount of 14 to 30% (Yamaguchi *et al*., 2004). However, such oil is susceptible to oxidation because of its high content of PUFAs; this oxidation could lead to its deterioration and off-flavor production (Nielsen *et al*., 2004). On the other hand, phenolic acids, which are known to be natural potent antioxidants, constitute another important group of compounds that may have beneficial effects in health (Silva *et al*., 2000; Torres de Pinedo *et al.,* 2007). Hence, lipase-catalyzed transesterification of fish liver oil with phenolic acids could produce structured lipids with phenolic moieties (Akoh *et al*., 1998). The synthesized phenolic lipids would have combined health benefits, antioxidant and improved solubility characteristics (Stamatis *et al*., 2001; Sabally *et al*., 2007).

This work is part of recent works in our laboratory, aimed at the development of lipase-catalyzed synthesis processes, for the production of selected structured phenolic lipids, with enhanced anti-oxidative and solubility properties (Lue *et al*., 2005; Sabally *et al.,* 2006 a,b; Safari *et al*., 2006; Sabally *et al.,* 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). However, the use of organic solvents may limit the acceptability of phenolic lipids as nutraceuticals and food ingredients (Martinez *et al*., 2004). In addition to providing clean media, the use of solvent-free medium (SFM) provides many other advantages, including the use of a smaller reaction volume, higher substrate concentrations, avoids the process of solvent-recovery as compared to organic solvent medium (OSM), as well as it is cost saving and an increase in the volumetric productivity can be achieved (Dossat *et al.,* 2002a; Fregolente *et al*., 2008). Previous work in our laboratory has demonstrated the efficiency of the biotechnological approach based on the use of SFM; for the synthesis of phenolic lipids using flaxseed oil and selected phenolic acids (Chapter III and IV). Different reaction parameters were optimized, including water activity, agitation speed, substrate and enzyme concentrations. A higher volumetric productivity (11-fold) in SFM was obtained as compared to that in OSM (Karboune *et al*., 2008). To expand the use of SFM for the enzymatic synthesis of novel phenolic lipids, the present work was aimed at the investigation of lipase-catalyzed transesterification of fish liver oil with selected phenolic acids in SFM. The specific objectives were to investigate the effects of phenolic acid structure, surfactants and time course on the bioconversion yield of phenolic lipids. The molecular structures of the synthesized

phenolic lipids and their radical scavenging activities were characterized. The effect of phenolic acid incorporation into the oil on its oxidative stability was also investigated.

5.3. Materials and Methods

5.3.1. Materials

Commercial immobilized lipase from *Candida antarctica* (Novozym 435, with an activity of 10,000 propyl laurate units, PLU, per g solid enzyme) was obtained from Novozymes Nordisk A/S (Bagsværd, Denmark). Fish liver oil, selected phenolic compounds, including caffeic, cinnamic, dihydrocaffeic, 3,4-dihydroxyphenyl acetic, ferulic and sinapic acids, as well as sodium methoxide, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), α-tocopherol, and surfactants, including Span 65 (Sorbitan tristearate) and Tween 65 (Polyoxyethylene Sorbitan tristearate), were purchased from Sigma Chemical Co. (St-Louis, MO). Gas liquid chromatography (GLC) standards were purchased from Nu-check Prep (Elysian, MN). Organic solvents of high-performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific (Fair Lawn, N.J.).

5.3.2. Transesterification Reaction in Solvent-Free Medium

Lipase-catalyzed transesterification of fish liver oil with selected phenolic acids, including caffeic, cinnamic, dihydrocaffeic, 3,4-dihydroxyphenyl acetic, ferulic and sinapic acids was carried out, according to the modification of Sabally *et al*. (2006b) method developed in our laboratory (Chapter III). Prior to each enzymatic reaction, a stock solution of phenolic acid (143 mM) in 2-butanone was freshly prepared. Aliquot of the phenolic acid stock solution was mixed with the fish liver oil to acquire a final concentration of 10 mM, where 2-butanone was 7% of the total reaction volume. The enzymatic reaction was initiated by the addition of 50 mg of solid enzyme (500 PLU) per mL reaction volume. The reaction mixture was incubated at 55°C, with continuous shaking at 150 rpm in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The transesterification reactions were run in duplicate in tandem with control trials that contain all components except the enzyme. The enzymatic reaction was monitored at selected time intervals over the course of 9 days. The recovered samples were flushed with a gentle stream of nitrogen and stored at - 80ºC for further analysis.

5.3.3. Characterization of the End Products

5.3.3.1. HPLC Analysis of the Reaction Components

HPLC analysis of the reaction components was carried out according to the method developed in our laboratory (Sabally *et al.,* 2007). The separation was performed on an Agilent Zobrax SB-C18 reversed-phase column (250x4.6 mm, 5 µm), using a Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). A volume of 20 µL sample was recovered and solubilized in 150 µL isopropanol. A volume of 20 µL of the diluted sample was subjected to HPLC analysis. The elution of the injected sample was carried out by a gradient solvent system, using methanol as solvent (A) and isopropanol as solvent (B). The elution was initiated by an isocratic flow of 100% of solvent A for 10 min, followed by a 10 min linear gradient to 40 and 60% of solvent A and B, respectively, then to 100% of solvent B for 10 min period. The elution was maintained for an additional period of 5 min before reverting to the initial conditions (100% solvent A), followed with an equilibration period of 10 min for the next sample. The flow rate was at 1 mL/min and the detection was performed at 280 nm for monitoring the phenolic lipids products.

The maximal bioconversion yield of phenolic lipids was calculated as the total peak area of phenolic lipids, detected at 280 nm, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100. The enzymatic activity was calculated from the slope of the linear portion of the plot of bioconversion yield versus the reaction time and it was expressed as nmol of PLs per g solid enzyme per min of reaction. The bioconversion yield of phenolic monoacylglycerols was defined as the peak area of the phenolic lipids, detected at 280 nm and eluted between 3 and 9 min, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100. However, the bioconversion yield of phenolic diacylglycerols was defined by the peak area of the phenolic lipids, detected at 280 nm and eluted between 20 and 26 min, divided by the peak area of residual phenolic acid and that of total phenolic lipids, multiplied by 100.

5.3.3.2. Mass Spectrometry Analysis of End Products

The characterization of reaction components of the lipase-catalyzed transesterification reaction was also performed by HPLC interfaced to an atmospheric pressure chemical ionization-mass

spectrometry (APCI-MS). The APCI-MS system (Thermo Finnigan, San Jose, CA) was equipped with the Zorbax SB-C18 reversed-phase column as well as a Surveyor liquid chromatography pump, an autosampler and Xcalibur® system control software (version 1.3) for data acquisition and processing. The mass spectrometer was operated in positive ion mode with a collision energy source of 15 V. The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

5.3.4. Effect of Surfactants on the Bioconversion Yield

The effects of selected surfactants, including Span 65 and Tween 65, at a concentration 5 mM on the transesterification reaction were investigated.

5.3.5. Determination of Relative Fatty Acid Composition

Fatty acids composition of fish liver oil and total phenolic lipids, recovered by preparative HPLC analysis, was investigated using GLC. Prior to GLC analysis, 5 mg of recovered total phenolic lipids fractions was methylated according to the method of Rocha-Uribe and Hernandez (2004), using 60 μL of 2 M sodium methoxide in 20% methanol. The mixture was incubated in reciprocal shaking water-bath (Model 25, Precision Scientific, Chicago, IL) at 65ºC. After 20 min of incubation, 10% sulfuric acid solution prepared in absolute methanol was added, followed by its incubation at 85ºC for 30 min. The methylated fatty acids were recovered with hexane. The upper layer was collected and recovered for GLC analysis. The analysis of fatty acid methyl esters was carried out with Agilent 6890 chromatography system (Agilent Technologies, Wilimington, DE), equipped with a FID detector, a split injector and HP-INNOWax polyethylene glycol fused capillary column (30 m × 0.25 mm I.D.× 0.25 μm film thickness). The injector and detector temperatures were set at 250°C. The column temperature was maintained at 150°C for 1 min and then increased to 200°C (10°C/min), which was held for 6 min followed by an additional increase to 220°C and was held for 6 min before it was increased to 225°C over a period of 5 min. The helium was used as the carrier gas, with a flow rate of 1 mL/min and a pressure of 9.8 psi, whereas that of air and hydrogen was 400 and 40 mL/min, respectively. Relative fatty acid percentages were compared by paired t-tests at $P < 0.05$.

5.3.6. Determination of the Free Radical Scavenging Activity of Phenolic Lipids

The free radical scavenging activity of phenolic lipids was determined according to a modification of Silva *et al.* (2000) method, using DPPH$^{\bullet}$ as the stable free radical compound. Prior to the analysis, a stock solution of DPPH$^{\bullet}$ (0.14 mM) was freshly prepared in absolute ethanol. The transesterified phenolic acids were recovered upon hydrolysis of phenolic lipids with 4 M NaOH for 4 h (Sosulski *et al*., 1982) and their concentrations were quantified, using Folin-Ciocalteu method (Singleton, 1965).

The investigated phenolic lipids as well as references were solubilized in the ethanolic solution of DPPH$^{\bullet}$ to yield a final concentration of (10, 20, 30, 50, 75, 100 and 150 µM). The decrease in DPPH$^{\bullet}$ concentration was followed spectrophotometrically with Beckman spectrophotometer (Model 650, Inc., Fullerton, CA) at 517 nm over a period of 80 min, using a blank assay without DPPH$^{\bullet}$. In addition, a control reaction containing only DPPH$^{\bullet}$ free radical was carried out in tandem with the scavenging reaction trial. The radical scavenging activity of the investigated compounds were compared on the basis of IC_{50}, which represents the concentration needed to reduce 50% of the initial amount of DPPH$^{\bullet}$ expressed in µM. Scavenging activity (SA) % versus sample concentration (SC) curve was plotted and the IC_{50} was calculated by non linear regression using SigmaPlot Program (version 11, Jandel Scientific, Germany). Data were found to fit the curve equation: $[SA\ \%] = A.e^{\,b[SC]}$. All trials were performed in triplicate and the percentage of residual DPPH$^{\bullet}$ was calculated using the absorbance of the reaction mixture at 517 nm (A_{max}), at a defined time, divided by the initial absorbance of DPPH$^{\bullet}$ control (A_i), multiplied by 100. The scavenging DPPH$^{\bullet}$ percentage was determined by subtracting the residual DPPH$^{\bullet}$ percentage from 100, multiplied by 100, where, A_{max} is the maximal absorbance of the phenolic products mixture after it reached the steady state, and A_i is the initial absorbance of the DPPH$^{\bullet}$ control trial.

5.3.7. Determination of the Oxidative Stability of Fish Liver Oil and Synthesized Phenolic Lipids

Three sets of unmodified fish liver oil and phenolic lipids samples were subjected to different treatments. The first set was incubated at 55ºC for 7 days, with continuous agitation at 150 rpm in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The second set was placed in open container at room temperature (25ºC) and exposed to light for 7 days, using a

daylight lamp (130 V, 60 W) with no shaking; whereas the third one was kept for 7 days in dark at room temperature with no shaking. Aliquots of each replicate were removed every other day for the determination of peroxide value (PV) according to the AOCS method (1989) as follows: 5 g sample was weighed into an Erlenmeyer flask, with 30 mL acetic acid-chloroform (3:2) solution. The sample was dissolved by swirling the flask, and then 0.5 mL of saturated potassium iodide solution was added. Solution was allowed to stand with occasional swirling for one minute before adding 30 mL distilled water. Slow titration with 0.01 N $Na_2S_2O_3$ was done until the color changes to light yellow. 0.5 mL of 1% soluble starch indicator was added, and then continuous titration while shaking until the blue color just disappears. PV was calculated as meq. of peroxide/kg of oil, equal to S x N x 1000/g weight of sample, where S is the volume of $Na_2S_2O_3$ in mL and N=0.01 is the concentration of the $Na_2S_2O_3$ solution.

5.3.8. Statistical Analysis

All experiments were analyzed as completely randomized design using PROC ANOVA of the statistical analysis system (SAS, 2009). Multiple comparisons of mean values were done by Tukey's honest significance test at $P < 0.05$.

5.4. Results and Discussion

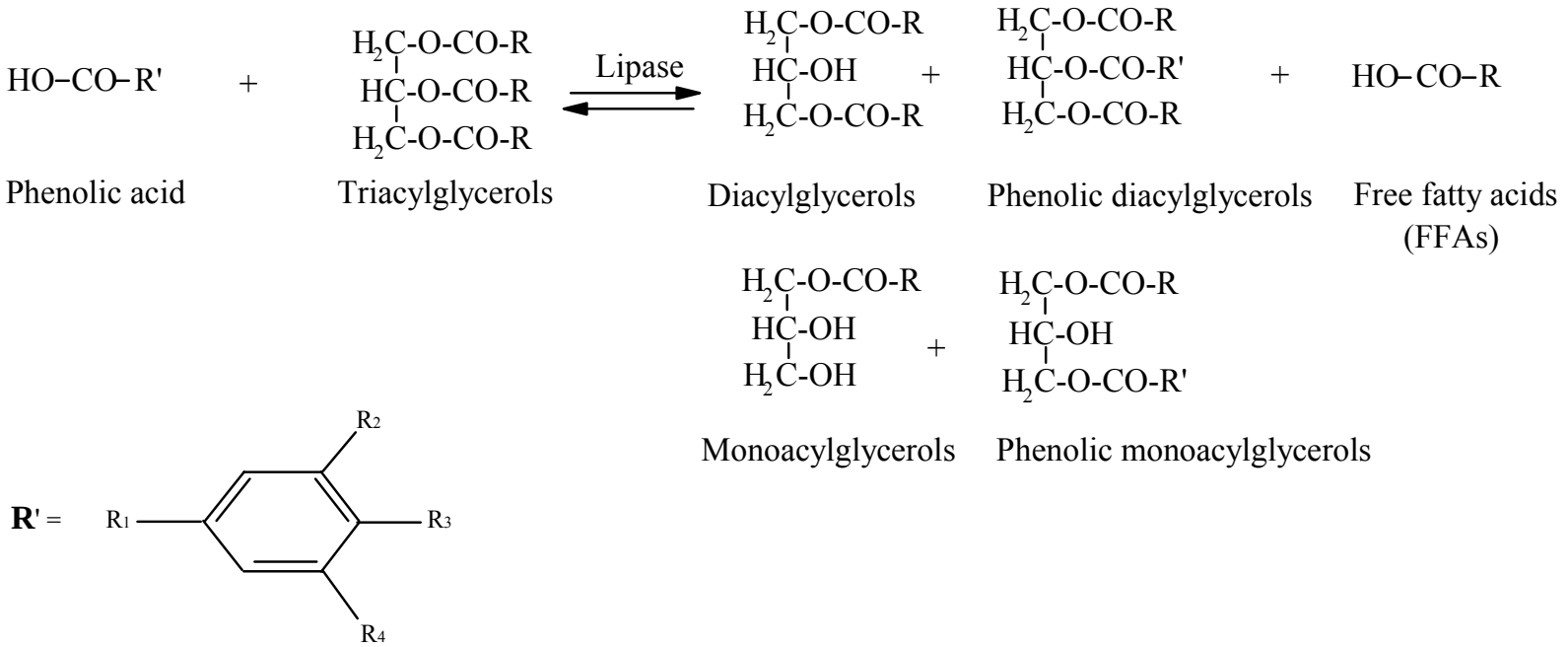
5.4.1. Effect of Phenolic Acid Structure on the Bioconversion Yield

The enzymatic synthesis of phenolic lipids in SFM obtained by the transesterification of fish liver oil with selected phenolic acids was investigated, using Novozym 435 from *Candida antarctica* as the biocatalyst (Fig. 19). The highest bioconversion yield (86%) was obtained with cinnamic acid at the 7^{th} day of the enzymatic reaction (Fig. 20). However, the *p*-hydroxylation of its aromatic ring (dihydrocaffeic and 3,4-dihydroxyphenyl acetic acids) resulted in a significant decrease ($P < 0.05$) in the bioconversion yield to 58 and 61%, respectively. While additional methoxylation of its aromatic ring (ferulic and sinapic acids) led to a further decrease in the bioconversion yield to 41 and 39%, respectively (Fig. 20). The inhibitory effects of the hydroxyl and methoxyl substituents of cinnamic acid derivatives on the enzyme activity have been previously reported (Buisman *et al*., 1998; Safari *et al*., 2006); this inhibitory effect was attributed to their electronic donating effects and/or to their steric hindrances at the enzyme active site. The presence of two *p*-hydroxyl groups on the benzene ring of caffeic acid significantly decreased ($P < 0.05$) the bioconversion from 86 to 39% as compared to that with

cinnamic acid. Similarly, Karboune *et al*. (2008) reported that the bioconversion yield was decreased significantly from 74 to 11%, with the use of caffeic acid and flaxseed oil in OSM. However, the lack of double bond in the side-chain, conjugated with the benzene ring of 3,4-dihydroxyphenyl acetic acid (DHPA) and dihydrocaffeic acid (DHCA) significantly decreased ($P < 0.05$) the inhibitory effect of the *p*-hydroxylation of the benzene ring, where 58 and 61% bioconversion yield was obtained, respectively (Fig. 20). Similar results were reported by Karboune *et al*. (2008) for the transesterification of DHPA with flaxseed oil in OSM as well as in SFM (Chapter 3), where 56 and 65% bioconversion yield were obtained, respectively. The overall experimental findings suggest that the inhibitory effect of *p*-hydroxyl substituents may be due to their electronic donating effects rather than to their steric hindrances (Buisman *et al*., 1998). Likewise, the transesterification of selected phenolic acids with flaxseed oil in SFM (Chapter 3) resulted in 87% bioconversion yield, with cinnamic acid as substrate; however, the *p*-hydroxylation of its aromatic ring as DHCA and DHPA resulted in a decrease in the bioconversion yield to 58 and 65%, respectively. In addition, the methoxylation of cinnamic acid aromatic ring as sinapic, 3,4-dimethoxybenzoic and ferulic acids resulted in a decrease in the bioconversion yield to 55, 57 and 49%, respectively. These results suggested that the effect of methoxyl and hydroxyl groups on the enzyme activity in SFM using flaxseed oil is somehow comparable (Chapter 3).

In contrast to the use of SFM as the reaction medium, a higher significant effect of the chemical structure of phenolic acids on the bioconversion yield was reported in OSM for the transesterification of flaxseed oil with selected phenolic acids (Karboune *et al.,* 2008). The difference between the two media may be attributed to the variations in their polarity and viscosity (Karboune *et al.,* 2005). The hydrophobic SFM medium containing fish liver oil may have increased the structural rigidity of lipase conformation as compared to that in OSM, decreasing hence the phenolic acid effect (Fitzpatrick and Klibanov, 1991; Dordick, 1992).

The time course (Fig. 20) for the enzymatic synthesis of selected phenolic lipids over a 9-day period of lipase-catalyzed transesterification of fish liver oil with selected phenolic acids, including caffeic, cinnamic, DHCA, DHPA, ferulic and sinapic acids, was investigated. The overall results show that the enzymatic synthesis of phenolic lipids was initiated rapidly within



R = Fish liver oil FFAs ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:5}$ and $C_{22:6}$)

Cinnamic acid : R_1 = CH = CH, R_2 = H, R_3 = H, R_4 = H

Ferulic acid : R_1 = CH = CH, R_2 = H, R_3 = OH, R_4 = O-CH_3

3,4-Dihydroxyphenyl acetic acid : R_1 = CH_2, R_2 = H, R_3 = OH, R_4 = OH

Dihydrocaffeic acid : R_1 = CH_2-CH_2, R_2 = H, R_3 = OH, R_4 = OH

Caffeic acid : R_1 = CH- CH, R_2 = H, R_3 = OH, R_4 = OH

Sinapic acid : R_1 = CH = CH, R_2 = O-CH_3, R_3 = OH, R_4 = O-CH_3

Figure 19. Reaction scheme of lipase-catalyzed transesterification reactions of selected phenolic acids with fish liver oil.

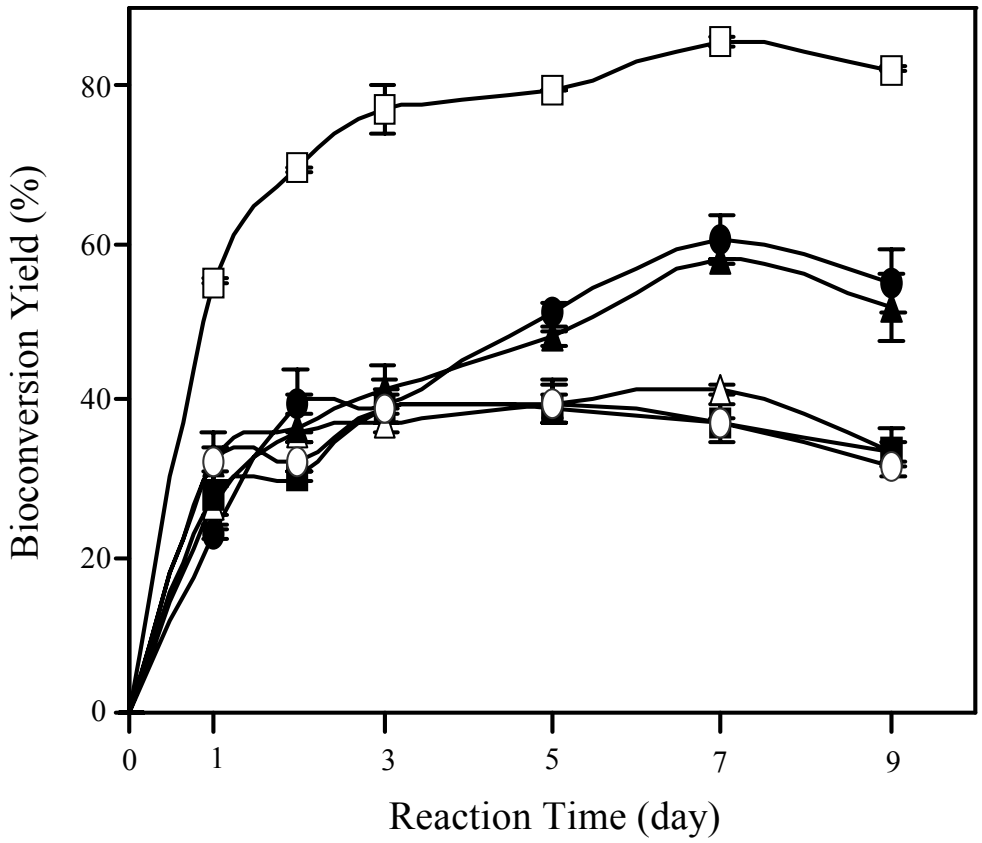


Figure 20. Time course of transesterification reaction using fish liver oil and selected phenolic acids in solvent-free medium, including caffeic acid (○), cinnamic (□), dihydrocaffeic acid (●), 3;4-dihydroxyphenyl acetic acid (▲), ferulic acid (△) and sinapic acid (■), STD ≤ 5.0.

the first day of the reaction, and then proceeded with different reaction rates over the time course. Similar trend was also reported (Sabally *et al.*, 2007; Karam *et al*., 2009) for lipase-catalyzed transesterification of fish liver oil with DHCA and DHPA in OSM. The highest bioconversion yield of 41, 61, 58 and 86% was obtained after 7 days of reaction with ferulic, DHCA, DHPA and cinnamic acids, respectively, before its decrease after additional 2 days to 33, 55, 52 and 82%, respectively. Likewise, Sabally *et al.* (2007) reported a 65% bioconversion yield by lipase-catalyzed transesterification of DHCA with fish liver oil in OSM, after 8 days of reaction. On the other hand, both caffeic and sinapic acids showed a 39% bioconversion yield after 5 days of reaction, and then decreased slightly to 31% after additional 4 days of reaction. The decrease in bioconversion yield may be due to a shift in the thermodynamic equilibrium of the reaction toward the hydrolysis and/or to the acyl migration of phenolic group (Karboune *et al.,* 2008).

5.4.2. Structural Characterization of Phenolic Lipids

Figure 21 shows the HPLC elution profile of the reaction components of lipase-catalyzed transesterification reactions of fish liver oil with DHCA, monitored at 280 nm. Peak # 1 (Figs. 21A and 21B), which absorbs at 280 nm, was characterized as DHCA. Six predominant peaks (2, 4, 5, 6, 7 and 8) were characterized as the main phenolic lipids end products of the transesterification reaction, since they have shown a UV-spectral scanning profile comparable to that of its phenolic acid component (Peak # 1). Triacylglycerols (TAGs) of fish liver oil were identified as peaks # 9 to 16 in the elution profiles of the reaction components (Figs. 21A and 21B).

In order to characterize the molecular structure of phenolic lipids, further analysis by HPLC/APCI-MS spectrometry of the eluted peaks were conducted. The phenolic group substitution on the glycerol backbone of the phenolic lipids is shown for simplicity, as the regioselectivity of the reaction is unknown (Karboune *et al.,* 2008). The fragmentation patterns of the main phenolic lipid products are shown in Figure 22. The analysis of peak #2 (Fig. 21B) resulted in a molecular ion of m/z 503.4 $[M+H–H_20]^+$ corresponding to monooleyl dihydrocaffeate, with a monoolein $[M+H\text{-}H_2O]^+$ fragment ion of *m/z* 339.4 (Fig. 22A). Figure 22B represents the analysis of peak #4 (Fig. 21B), confirming hence the synthesis of a

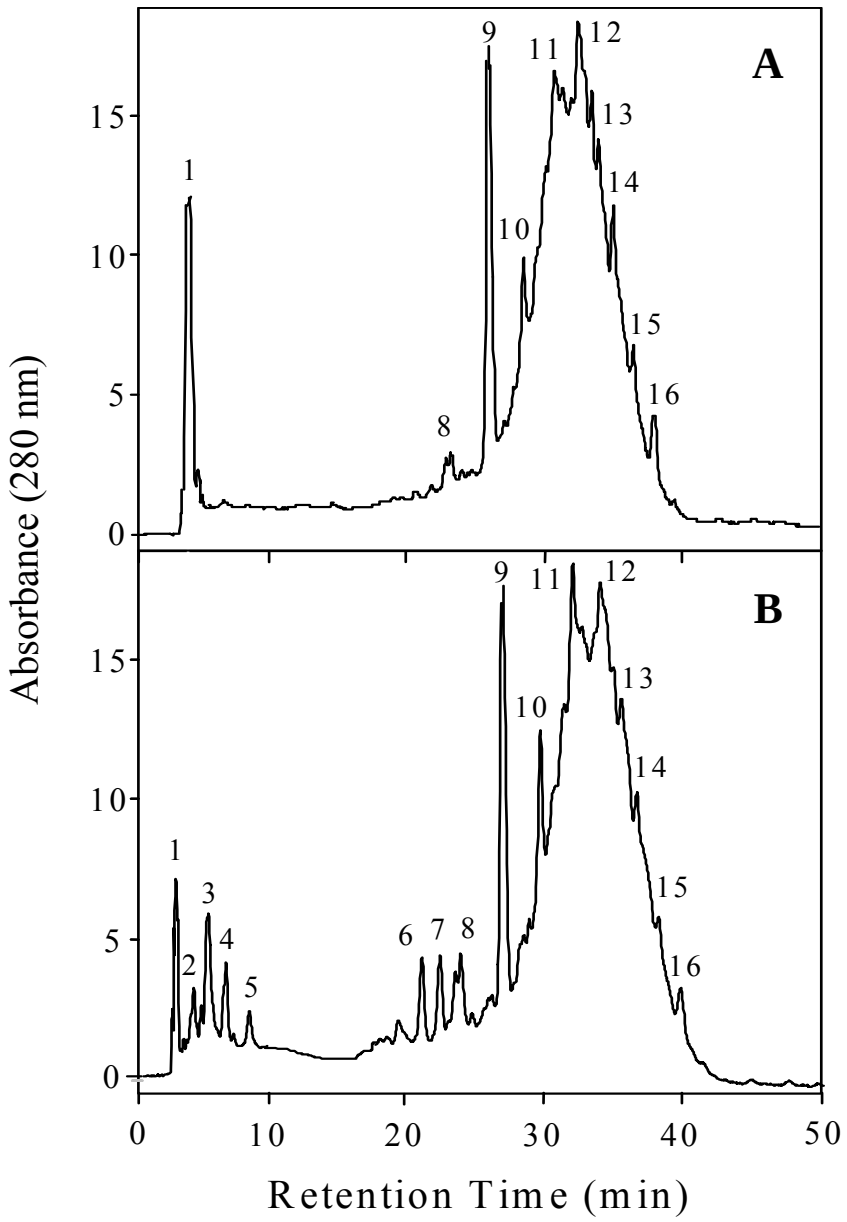


Figure 21. HPLC chromatograms of the initial reaction mixture of dihydrocaffeic acid (DHCA) with fish liver oil (A), the enzymatic transesterification reaction mixture (B) monitored at 280 nm. Peak numbers were identified as follows: DHCA # 1, monoacylglycerols # 3, phenolic monoacylglycerols # 2, 4, 5, phenolic diacylglycerols # 6, 7, 8 and fish liver oil # 9, 10, 11, 12, 13, 14, 15 and 16.

monoeicosapentaenoyl dihydrocaffeate with a molecular ion $[M+H]^+$ of *m/z* 541.3 and a monoeicosapentaenoin $[M+H–H_2O]^+$ fragment ion of *m/z* 359.5. While the analysis of peak #5 (Fig. 21B) showed a molecular ion of *m/z* 567.6 $[M+H]^+$ corresponding to monodocosahexaenoyl dihydrocaffeate and a $[M+H–H_2O]^+$ fragment ion of *m/z* 385.4 corresponding to monodocosahexaenoin (Fig. 22C). The analysis of peak #6 (Fig. 21B) confirmed the synthesis of a dieicosapentaenoyl dihydrocaffeate, with a molecular ion $[M]^+$ of *m/z* 824.7 and a fragment ion $[M+H–H_2O]^+$ of *m/z* 643.8 representing dieicosapentaenoin (Fig. 22D). In addition, the fragmentation of peak #7 (Fig. 21B) resulted in a characteristic pattern of eicosapentaenoyl docosahexaenoyl dihydrocaffeate (Fig. 22E), with an abundant molecular ion at *m/z* of 850.1 $[M]^+$ and fragment ion $[M+H–H_2O]^+$ at 669.6 corresponding to eicosapentaenoin docosahexaenoin glyceride. While the fragmentation of peak #8 (Fig. 21B) produced a mixed phenolic diacylglycerol molecular ion $[M]^+$ at *m/z* 876.0 being didocosahexaenoyl dihydrocaffeate (Fig. 22F), with a fragment ion $[M+H–H_2O]^+$ of *m/z* 694.7 corresponding to didocosahexaenoin. The loss of H_2O from a monoacylglycerol during HPLC/APCI-MS analysis has been previously reported (Sabally *et al.,* 2007; Karam *et al.,* 2009).

In summary, the HPLC/APCI-MS analysis confirmed the formation of 3 phenolic monoacylglycerols and 3 phenolic diacylglycerols by the lipase-catalyzed transesterification of fish liver oil with DHCA in SFM. However, Sabally *et al.* (2007) has characterized 2 phenolic monoacylglycerols (monopalmityl dihydrocaffeate and monooleyl dihydrocaffeate) and 2 phenolic diacylglycerols (dieicosapentaenoyl dihydrocaffeate and eicosapentaenoyl docosahexaenoyl dihydrocaffeate), using fish liver oil and DHCA in OSM. In addition, Karam *et al.* (2009) reported the characterization of 8 dihydroxyphenyl acetoylated lipids as the end products of lipase-catalyzed transesterification of fish liver oil with DHPA, in OSM and 3 of them were phenolic diacylglycerols.

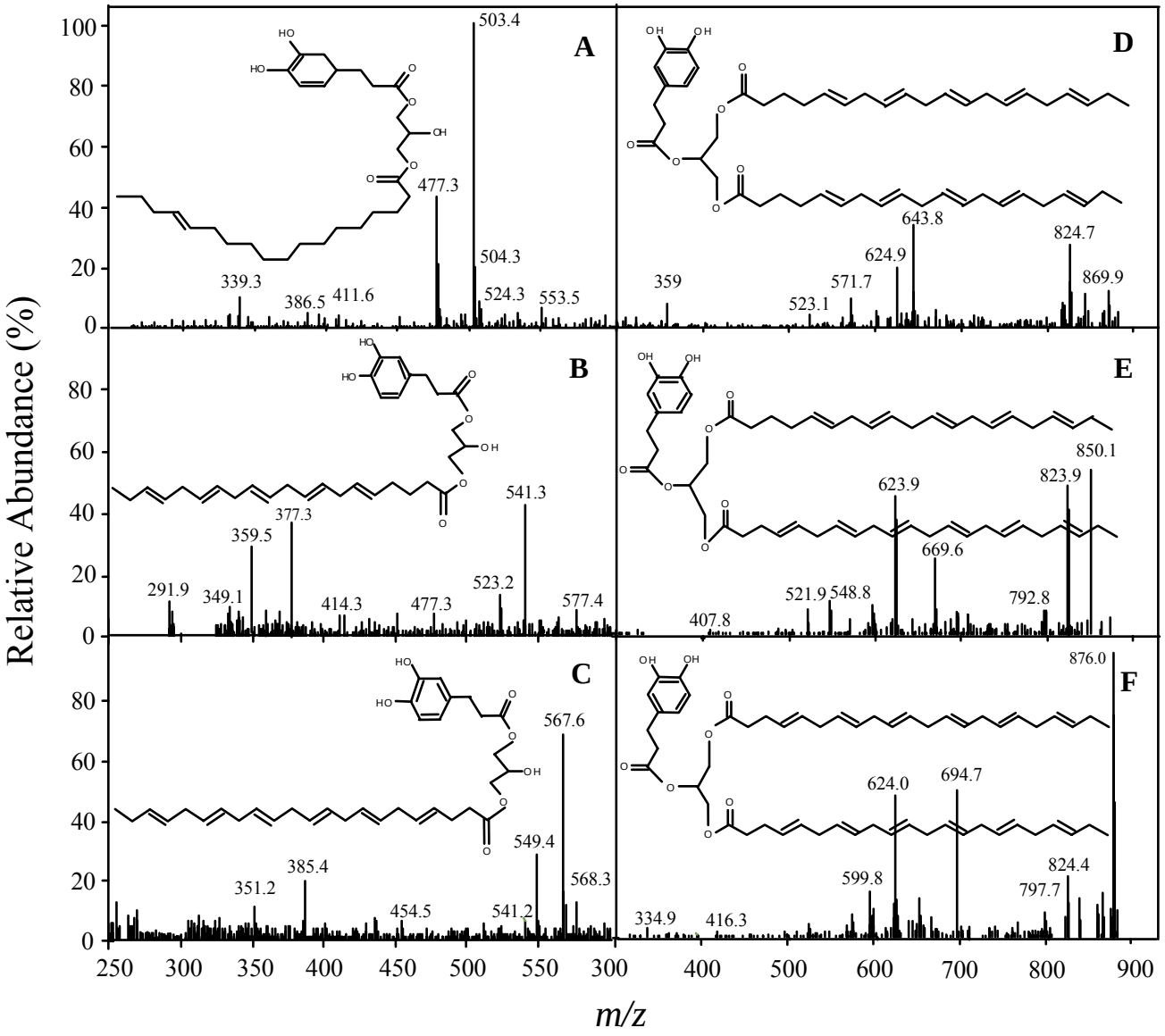


Figure 22. Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) analysis of dihydrocaffeoylated monoacylglycerol (A, B, C), -diacylglycerols (D, E and F).

5.4.3. Effect of Surfactants on the Bioconversion Yield

Because of the ability of surfactants to decrease the surface tension, they increase the interfacial area between substrates and enzyme molecules; such effect may harbor more lipase molecules, where the increase in the solubility of substrates can consequently increase the rate of the enzymatic reaction (Liu *et al*., 2000). The effect of selected surfactants, including non-ionic Span 65 and Tween 65, on the enzyme activity and the bioconversion yield of phenolic lipids in SFM was investigated (Table 9; Fig. 23). The addition of Span 65 and Tween 65 (Table 9) didn't result in a similar effect on the enzymatic activity of the lipase-catalyzed transesterification of selected phenolic acids. However, a significant increase ($P < 0.05$) in the enzymatic activity from 177 to 240 and 235 nmol of PLs/g solid enzyme/min was obtained upon the addition of Span 65 and Tween 65, respectively, using DHCA; similarly, using DHPA as phenolic acid an increase from 144 to 206 and 189 nmol of PLs/g solid enzyme/min was observed. Likewise, the addition of Span 65 to the lipase-catalyzed transesterification of flaxseed oil and DHPA (Chapter 3) resulted in a significant increase ($P < 0.05$) in the enzyme activity up to 270 nmol of PLs/g solid enzyme/min as compared to that in the control trial without surfactant (200 nmol of PLs/g solid enzyme/min).

In contrast, Liu *et al*. (2000) reported a higher enzymatic activity in OSM, up to 13 times for *Candida rugosa* lipase, coated with the non-ionic surfactant Tween-80, as compared to the one coated with anionic or cationic surfactants. In addition, Goto *et al*. (1994) suggested that the interaction between the non-ionic surfactant and the lipase via a weak interaction would favor the maintenance of the stereoconfiguration of lipase for better catalytic performance, which would result in a high bioconversion yield. The overall experimental findings (Table 9) indicate that the effect of surfactant on the enzymatic activity in SFM was not highly significant as compared to that performed in OSM or in buffer medium (Kamiya *et al.,* 1995; Goto *et al.,* 1994; Liu *et al*., 2000). These results (Table 9) may be attributed to the nature of the SFM in terms of viscosity and polarity, which would have affected the interactions between the lipase and the surfactants. The hydrophobic SFM medium containing fish liver oil may have increased the structural rigidity of lipase conformation, as compared to OSM, which could result in a less effect of the surfactants (Fitzpatrick and Klibanov, 1991; Dordick, 1992).

Table 9. Effect of selected surfactants on the enzyme activity of lipase-catalyzed transesterification of fish liver oil with selected phenolic acids in solvent-free medium[a].

	Enzyme activity[b]		
Phenolic compound	Control[c]	Span 65	Tween 65
Caffeic acid	134 (2.0)[d]	149 (1.2)[d]	136 (3.1)[d]
Cinnamic acid	190 (4.2)[d]	196 (3.3)[d]	198 (4.2)[d]
Dihydrocaffeic acid	177 (2.5)[d]	240 (0.7)[d]	235 (3.4)[d]
3,4-Dihydroxyphenyl acetic acid	144 (3.8)[d]	206 (4.5)[d]	189 (7.4)[d]
Ferulic acid	104 (3.8)[d]	124 (3.4)[d]	115 (3.6)[d]
Sinapic acid	105 (6.0)[d]	122 (3.5)[d]	120 (2.0)[d]

[a]Transesterification reaction of fish liver oil and selected phenolic acids was carried out in solvent-free medium in the presence of surfactants, including Span 65 and Tween 65 (5 mM) for 9 days reaction period.

[b]Enzyme activity is defined as nmol of PLs/g solid enzyme/min reaction time.

[c]Control trial; without surfactant.

[d]Data are average of duplicate samples, and the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

Figure 23 shows that the trend of the enzymatic synthesis of phenolic lipids with and without surfactants were similar throughout the 9-days reaction period. However, the reaction rate was higher with surfactants addition, where the maximum bioconversion yield (59%) was reached after 5 days of reaction as compared to 61% obtained at day 7 in the control trial. The addition of Span and Tween resulted in a maximum bioconversion yield of 10 and 12%, respectively, of dihydrocaffeoylated monoacylglycerols after 5 days of reaction, as compared to 12% at day 7 of reaction in the control trial. While, the bioconversion yield of dihydrocaffeoylated diacylglycerols increased rapidly within the first 2 days to 42 and 35%, respectively, and to a lower extent thereafter up to a maximum of 49 and 44% after 5 days of reaction, as compared to 49% at day 7 of reaction in the control trial. However, it decreased to 40, 36 and 44%, with Span, Tween and control, respectively, after 9 days of reaction. This decrease may be due to a shift in the thermodynamic equilibrium of the reaction toward hydrolysis and/or to the acyl migration of the phenolic group (Karboune *et al.,* 2008).

In contrast to OSM (Sabally *et al.,* 2007; Karam *et al.,* 2009), lipase-catalyzed transesterification of fish liver oil with selected phenolic acids in SFM was more in favor of the synthesis of phenolic diacylglycerols. The biocatalysis in OSM, using fish liver oil and DHCA showed a bioconversion yield of dihydrocaffeoylated mono- and diacylglycerols up to a maximum of 29 and 37%, respectively (Sabally *et al.,* 2007), whereas the use of SFM (Fig. 23) resulted in a bioconversion yield of 12 and 49%, respectively. The difference in reaction selectivity between the OSM and SFM may be attributed to their micro-environment effect affecting differently the substrate availability (Karboune *et al.,* 2005; Chapter III).

On the basis of the experimental findings, the addition of Span 65 to the reaction medium showed its beneficial effect on the reaction rate for the enzymatic synthesis of phenolic lipids from fish liver oil and DHCA.

5.4.4. Determination of Relative Fatty Acid Composition

The fish liver oil contains about 21 different fatty acids (Sabally *et al.,* 2007). Among the 11 major fatty acids (FAs), indicated in Table 10, 5 major FAs were determined to be $C_{16:0}$ (10.5%),

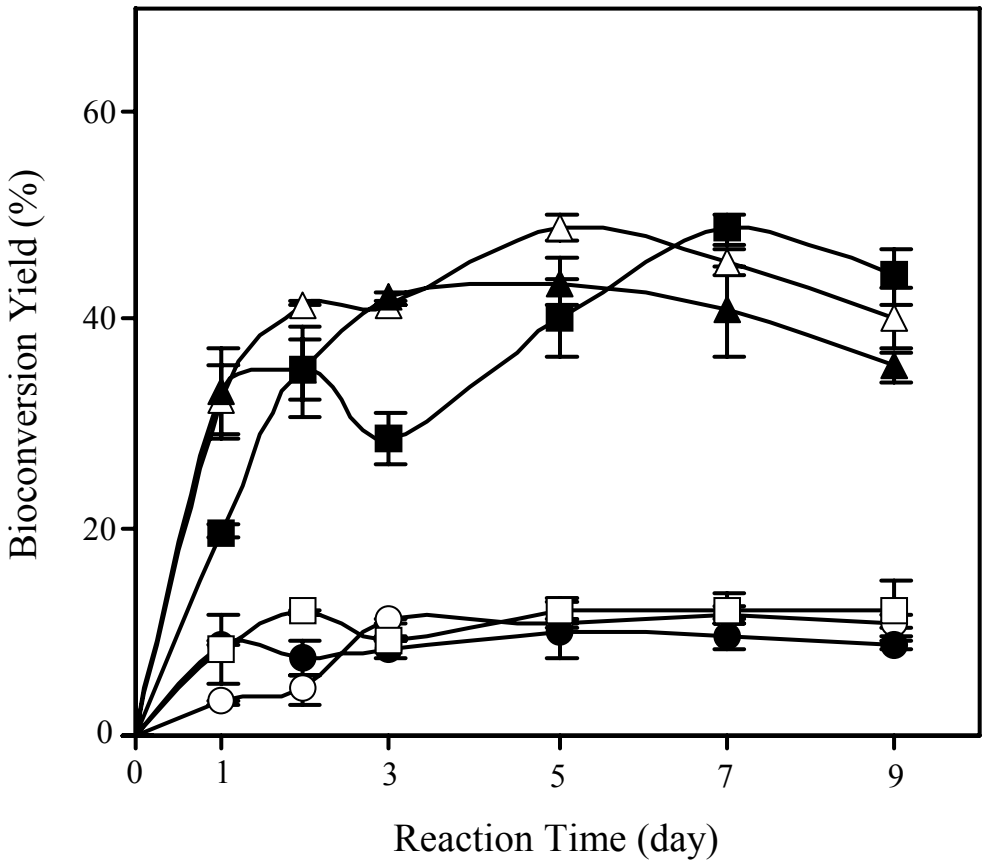


Figure 23. Time course for the transesterification reaction of dihydrocaffeic acid with fish liver oil in solvent-free medium: phenolic monoacylglycerols without surfactant (○), phenolic monoacylglycerols with Span (●), phenolic monoacylglycerols with Tween (□), phenolic diacylglycerols without surfactant (■), phenolic diacylglycerols with Span (△) and phenolic diacylglycerols with Tween (▲) addition, STD ≤ 4.7.

$C_{18:1}$ *n*-9 (16.5%), $C_{20:1}$ *n*-9 (11.0%) $C_{20:5}$ *n*-3 (11.5%) and $C_{22:6}$ *n*-3 (12.0%). As a result of the transesterification of fish liver oil with the selected phenolic acids, including DHCA, DHPA, caffeic, ferulic and sinapic acids, the relative proportions of the two highly desirable essential fatty acids (EPA, $C_{20:5}$ *n*-3) and (DHA, $C_{22:6}$ *n*-3) were significantly increased ($P < 0.05$). EPA was increased from 11.5% in the fish liver oil to 21.2, 20.7, 20.8, 20.1 and 19.8%, respectively, in dihydrocaffeoylated, 3,4-dihydroxyphenyl acetoylated, caffeoylated, feruloylated and sinapoylated lipids, while DHA was increased from 12.0% in the fish liver oil to 21.4, 19.4, 27.5, 22.1 and 22.0%, respectively. The percent of shorter-chain FAs in the total phenolic lipids, was decreased after transesterification, where $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ decreased from 4.0, 10.5 and 2.5% in the fish liver oil to 2.6, 6.5 and 1.6%, respectively, in dihydrocaffeoylated lipids (Table 10). However, $C_{18:3}$ *n*-3 manifested a slight increase from 1.0% in the crude fish liver oil to 1.5% in dihydrocaffeoylated lipids. Similar results were obtained upon the transesterification of DHPA and fish liver oil in OSM (Karam *et al*., 2009). In addition, all investigated total phenolic lipids followed similar trend (Table 10).

The overall results (Table 10) suggest that Novozym 435 has a lower specificity and stereoselectivity towards the exchange of the $C_{20:5}$ *n*-3 and $C_{22:6}$ *n*-3 acyl groups with that of the phenolic acid, as compared to those of $C_{14:0}$, $C_{16:0}$ (Ramirez *et al.*, 2003; Karam *et al*., 2009). Similar results were obtained upon the transesterification of DHPA and flaxseed oil in SFM (Chapter IV) as well as for the transesterification of DHPA and fish liver oil in OSM (Karam *et al*., 2009). In contrast, Sabally *et al.* (2007) reported a decrease in the proportions of longer-chain FAs and an increase in the shorter-ones in the phenolic mono- and diacylglycerols, upon the transesterification of DHCA and fish liver oil in OSM. These findings could have revealed that carrying out the enzymatic reaction in SFM, with an excess amount of fish liver oil in the reaction mixture; showed a higher specificity towards the synthesis of the more non-polar shorter-chain FAs (Karam *et al*., 2009). Likewise, Hills *et al.* (1990) reported a decrease in the proportion of $C_{18:2}$ FA and a 2-fold increase in the longer-chain FAs, $C_{22:5}$ and $C_{20:6,}$ upon the esterification of cod liver oil and primrose oil.

Table 10. Relative fatty acid composition of fish liver oil before and after its transesterification with selected phenolic acids in solvent-free medium.

	Relative fatty acid (%)[a]					
Fatty acid	Fish liver oil	DHCA lipids[b]	DHPA lipids[b]	CAF lipids[b]	FER lipids[b]	SIN lipids[b]
$C_{14:0}$	4.0 (3.5)[c]	2.7 (1.6)[c]	2.5 (3.5)[c]	2.7 (1.7)[c]	2.3 (1.4)[c]	2.2 (1.5)[c]
$C_{16:0}$	10.5 (2.7)[c]	6.5 (5.8)[c]	7.0 (1.4)[c]	6.8 (3.6)[c]	5.6 (1.8)[c]	4.5 (1.2)[c]
$C_{16:1}$	8.0 (3.2)[c]	8.0 (1.5)[c]	7.4 (2.5)[c]	7.3 (3.5)[c]	8.0 (2.8)[c]	7.5 (4.5)[c]
$C_{18:0}$	2.5 (2.5)[c]	1.6 (1.5)[c]	1.1 (2.2)[c]	1.7 (4.8)[c]	1.0 (2.6)[c]	0.9 (3.3)[c]
$C_{18:1}$ *n-9*	16.5 (5.2)[c]	10.6 (8.1)[c]	14.9 (8.1)[c]	10.4 (8.9)[c]	11.9 (2.2)[c]	13.0 (2.7)[c]
$C_{18:2}$ *n-6*	1.7 (3.5)[c]	2.2 (1.6)[c]	0.9 (3.9)[c]	0.7 (5.7)[c]	0.9 (4.7)[c]	1.1 (3.1)[c]
$C_{18:3}$ *n-3*	1.0 (3.2)[c]	1.5 (4.3)[c]	1.0 (4.9)[c]	1.5 (4.8)[c]	1.2 (3.3)[c]	1.4 (1.9)[c]
$C_{20:1}$ *n-9*	11.0 (2.5)[c]	13.0 (3.8)[c]	14.2 (2.5)[c]	12.5 (2.5)[c]	15.0 (1.4)[c]	15.6 (1.2)[c]
$C_{20:5}$ *n-3*	11.5 (2.5)[c]	21.2 (1.5)[c]	20.7(1.3)[c]	20.8 (6.6)[c]	20.1 (2.9)[c]	19.8 (3.0)[c]
$C_{22:1}$ *n-11*	8.5 (1.5)[c]	11.2 (3.7)[c]	10.8 (1.5)[c]	8.0 (3.1)[c]	12.0 (2.2)[c]	12.0 (2. 3)[c]
$C_{22:6}$ *n-3*	12.0 (3.2)[c]	21.4 (3.4)[c]	19.4 (3.5)[c]	27.5 (2.4)[c]	22.1 (3.7)[c]	22.0 (1.2)[c]

[a]Relative fatty acid (%) was calculated was calculated as the peak area of each fatty acid divided by the total peak area of all fatty acids, multiplied by 100.

[b]Phenolic lipids, include dihydrocaffeoylated (DHCA lipids), 3,4-dihydroxyphenyl acetoylated (DHPA lipids), caffeoylated (CAF lipids), feruloylated (FER lipids) and sinapoylated (SIN lipids).

[c]Relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

5.4.5. Determination of the Radical Scavenging Activity of Phenolic Lipids

Among the naturally occurring phenolic compounds, phenolic acids and their derivatives are recognized as natural potent antioxidants, exhibiting radical scavenging activity (Silva *et al.,* 2000). The radical scavenging activity of caffeic, DHCA, DHPA, ferulic, sinapic acids and their phenolic lipid esters toward a stable free radical DPPH$^{\bullet}$ was evaluated and compared with that of α-tocopherol. The results (Table 11) show that the steady state of the scavenging reaction of DPPH$^{\bullet}$ was reached after 3 to 75 min with all phenolic acids, 17.5 to 75 min with phenolic lipids and 1 to 24 min with α-tocopherol for the investigated concentrations. In addition, the IC_{50}, which represents the concentration needed to reduce 50% of the initial concentration of DPPH$^{\bullet}$, was calculated. The experimental findings (Table 11) indicated that the most potent radical scavenger was DHCA closely followed by DHPA, caffeic acid, sinapic acid, α-tocopherol and finally ferulic acid. The results (Table 11) indicate that DHCA possessed a higher scavenging capacity than caffeic acid which is in agreement with results reported by Silva *et al.* (2000), these results may be due to the fact that DHCA has a side chain connected to the aromatic ring by single bonds, which allows the phenyl group to have a certain flexibility to rotate, whereas caffeic acid has a coplanar conformation (Silva *et al.*, 2000). While, DHPA scavenging activity may be due to the presence of two hydroxyl groups, which is known to form quinone and increase the resonance stabilization (Brand-Willams *et al*., 1995; Chen and Ho, 1997). In addition, the presence of an alkenyl chain in some phenolic compounds, such as caffeic and sinapic acids, could be important in the stabilization of the formed radical (Nenadis *et al.,* 2003).

The overall experimental results (Table 11) are in agreement with those previously reported (Chen and Ho, 1997; Silva *et al.*, 2000; Nenadis *et al.,* 2003; Karboune *et al*., 2008), using the DPPH$^{\bullet}$ as the free radical. Despite the major advances in understanding the molecular mechanism underlying the radical scavenging activity, there are still some controversies regarding the relationship between the molecular structure of phenolic compounds and their reactivity as radical scavengers (Chen and Ho, 1997). However, the main structural characteristics of phenolic acids, required for an efficient radical scavenging activity, were reported to be the number of hydroxyl groups on the benzene ring being the most important (Chen and Ho, 1997; Silva *et al.,* 2000) as well as the number of the methoxyl groups.

Table 11. Radical scavenging effect of phenolic lipids and its corresponding phenolic acid.

Phenolic compound	Time to reach steady state (min)[a]	IC_{50}[b]
Caffeic acid	4.0 - 45.0	17.1 (3.3)[c]
Dihydrocaffeic acid	3.0 - 41.5	10.2 (8.3)[c]
3,4-Dihydroxyphenyl acetic acid	7.5 - 36.0	11.1 (8.5)[c]
Ferulic acid	36.0 - 75.0	37.6 (3.5)[c]
Sinapic acid	3.0 - 50.0	25.7 (8.5)[c]
Caffeoylated lipids	5.0 - 75.0	32.6 (2.6)[c]
Dihydrocaffeoylated lipids	17.5 - 47.5	27.3 (4.3)[c]
3,4-Dihydroxyphenyl acetoylated lipids	25.5 - 42.7	36.3 (3.1)[c]
Feruloylated lipids	32.0 - 64.0	61.5 (1.4)[c]
Sinapoylated lipids	45.0 - 75.0	54.7 (5.1)[c]
α-Tocopherol	1.5 - 24.0	34.4 (3.6)[c]

[a]Determination of the steady state at concentrations range (10-150 μM).

[b]IC_{50} represents the concentration of phenolic compounds in μM needed to reduce 50% of the initial amount of $DPPH^{\bullet}$.

[c]Data are average of three measurements and the relative standard deviations was calculated from the standard deviation of triplicate samples divided by their mean, multiplied by 100.

On the other hand, among the investigated phenolic lipids, dihydrocaffeoylated lipids showed the highest radical scavenging activity, where only 27.3 μM was required to reduce 50% of the initial amount of DPPH$^{\bullet}$ as compared to 34.4 μM of α-tocopherol, followed by 3,4-dihydroxyphenyl acetoylated lipids, with IC_{50} value of 36.3 μM. However, feruloylated lipids showed the lowest radical scavenging activity, with the highest significant ($P < 0.05$) IC_{50} value of 61.5 μM. Similarly, Chen and Ho, (1997) reported that the radical scavenging activity of caffeic acid and its ester was greater than that of ferulic acid and its ester, which is in agreement with the present results (Table 11).

The results obtained (Table 11) suggest that the transesterification of all phenolic acids reduced their scavenging activity by 1.6 to 3.3-fold, as the side-chain linked to the aromatic ring by a single bond that allows the phenyl group to have a certain flexibility to rotate (Silva *et al.*, 2000). The attachment of a mono- or diacyglycerol moiety to the side chain of phenolic acid may have reduced or restricted the rotation degree of the phenyl moiety along the side chain, leading to a reduction in its ability to scavenge free radical (Sabally *et al.,* 2007; Karam *et al*., 2009). In addition, the molecular conformation of phenolic acids could be one of the factors affecting their antiradical activity, which is intrinsically related to DPPH$^{\bullet}$ scavenging (Silva *et al.*, 2000). Similar results were reported by Karboune *et al*. (2008) and Karam *et al*. (2009) for the antioxidant activity of phenolic lipids obtained by the transesterification of selected phenolic acids in OSM with flaxseed and fish liver oils, respectively.

5.4.6. Determination of the Oxidative Stability of Fish Liver Oil and Synthesized Phenolic Lipids

In order to evaluate the oxidative stability of the phenolic lipids, the peroxide value (PV) of both unmodified fish liver oil and synthesized phenolic lipids, subjected to different storage conditions, was determined (Table 12). The PV of the untreated fish liver oil (2.3 meq peroxide/kg oil) was increased significantly ($P < 0.05$) to 16.2 meq peroxide/kg oil after 1 day of incubation at 55ºC and 150 rpm agitation speed. Then the PV gradually increased to 31.6 meq peroxide/kg oil after 7 days of incubation (Table 12). However, a significant increase ($P < 0.05$) in the oxidation to 221.2 meq peroxide/kg oil after 7 days of incubation was obtained when samples were incubated opened under the same conditions. However, samples exposed to light at room temperature without shaking, showed less oxidation with PV of 5.2 meq peroxide/kg oil

after 1 day of incubation before its significant increase ($P < 0.05$) to 38.8 meq peroxide/kg oil after 7 days. In addition, oil samples stored in dark at 25ºC showed less ($P < 0.05$) oxidation (17.4 meq peroxide/kg oil) after 7 days of storage as compared to those exposed to light treatment (38.8 meq peroxide/kg oil). The experimental data (Table 12) indicates that exposing oil samples to high temperature (55ºC) with agitation enhanced the rate of lipids oxidation; these results are in agreement with those obtained by Nielsen *et al.* (2003) for tuna oil oxidation. Likewise, Siriwardhana *et al.* (2004) reported that the aerated fish liver oil samples, incubated at 60ºC, showed a PV of 100 meq peroxide/kg oil after 8 days of incubation, and increased gradually to 260 meq peroxide/kg oil after an additional 4 days of incubation.

On the other hand, the synthesized phenolic lipids (Table 12) showed less susceptibility for oxidation, where the PVs were significantly ($P < 0.05$) lower than that of the unmodified fish liver oil for all treatments. The PV of the unmodified fish liver oil was significantly increased ($P < 0.05$) by 9.2-fold as compared to PV of 24.2 meq peroxide/kg oil obtained when the phenolic lipids samples were incubated opened for 7 days at 55ºC and 150 rpm agitation speed. In addition, light has less effect on the oxidation of phenolic lipids (11.4 meq peroxide/kg oil) as compared to that on the unmodified fish liver oil (38.8 meq peroxide/kg oil) after 7 days of incubation. The overall experimental data (Table 12) indicates that the synthesized phenolic lipids, which exhibited radical scavenging activity comparable to that of α-tocopherol (Sabally *et al*., 2007; Karam *et al.,* 2009), has the ability to reduce the oxidation more than the unmodified fish liver oil. Similarly, Akoh and Moussata (2001) reported that the addition of antioxidants, such as α-tocopherol and *tert*-butylhydroxyquinone, improved the oxidative stability of structured lipids, as compared to unmodified fish liver oil when opened samples were incubated at 60°C in a shaking water bath.

Both the radical scavenging activity and the PV demonstrated the antioxidant potential of phenolic lipids throughout their ability to scavenge the free radical by hydrogen donation and to limit the propagation step, respectively (AOCS, 1989; Silva *et al.,* 2000). In summary, determination of the PV could provide an indication of the oxidative status. Several studies have reported that the secondary oxidized oil products are generally toxic, and even weakly oxidized fat and oil at levels of PV equal to 100 meq/kg of oil are neurotoxic (Gotoh *et al.,* 2006).

Table 12. Temperature and light exposure effect on fish liver oil and the synthesized phenolic lipids oxidative stability.

	Peroxide value[a]									
	Fish liver oil					Phenolic lipids				
Treatment	Day 1	Day 2	Day 3	Day 5	Day 7	Day 1	Day 2	Day 3	Day 5	Day 7
1[b]	16.2 (1.3)[f]	20.9 (6.1)[f]	21.5 (2.9)[f]	21.9 (2.6)[f]	31.6 (1.8)[f]	2.9 (4.9)[f]	7.6 (6.6)[f]	9.3 (2.3)[f]	13.1 (1.1)[f]	20.3 (9.0)[f]
2[c]	25.7 (3.0)[f]	64.2 (0.1)[f]	75.3 (0.8)[f]	141.5 (1.5)[f]	221.2 (0.8)[f]	15.3 (2.3)[f]	15.5 (4.6)[f]	16.6 (0.4)[f]	22.2 (0.9)[f]	24.2 (2.1)[f]
3[d]	5.2 (9.6)[f]	7.4 (2.8)[f]	10.3 (3.4)[f]	21.0 (5.4)[f]	38.8 (1.6)[f]	1.7 (8.3)[f]	1.9 (7.4)[f]	2.7 (8.0)[f]	3.9 (5.5)[f]	11.4 (7.4)[f]
4[e]	4.6 (9.2)[f]	6.6 (4.3)[f]	8.0 (3.5)[f]	9.0 (2.4)[f]	17.4 (3.3)[f]	1.7 (6.3)[f]	1.7 (2.0)[f]	1.8 (7.9)[f]	3.2 (6.7)[f]	7.6 (1.9)[f]

[a]Peroxide value is determined according to Official Methods of Analysis of AOAC, and calculated as meq. of peroxide/kg of oil, S x N x 1000/weight of sample in grams, where S = mL of $Na_2S_2O_3$, and N= 0.01 is the concentration of the $Na_2S_2O_3$ solution.

[b]Closed samples were placed at (55ºC) with shaking at 150 rpm for 7 days.

[c]Opened samples were placed at (55ºC) with shaking at 150 rpm for 7 days.

[d]Opened samples were placed at room temperature and exposed to light for 7 days.

[e]Opened samples were placed at room temperature in dark for 7 days

[f]Data are average of duplicate samples, and the relative standard deviation was calculated from the standard deviation of duplicate samples divided by their mean, multiplied by 100.

Hence, the formation of hydroperoxide must be suppressed to protect the oxidation of fats and oils, from both food quality and food safety perspectives (Gotoh *et al*., 2006).

5.5. Conclusion

An environmentally safe and volume efficient process for the enzymatic synthesis of selected phenolic lipids from fish liver oil and DHCA was achieved in SFM. The modification process resulted in a significant increase in the relative proportions of the two highly desirable essential fatty acids, (EPA, $C_{20:5}$ *n*-3) and (DHA, $C_{22:6}$ *n*-3) in the synthesized phenolic lipids. In addition, the experimental data indicated that the synthesized phenolic lipids demonstrated radical scavenging activity compared to that of α-tocopherol, indicating their potential as antioxidants.

CHAPTER VI

STATEMENT OF CHAPTER VI LINKAGE

As a result of the research work conducted previously (Chapters V) on the enzymatic synthesis of phenolic lipids from fish liver oil using different phenolic acids, dihydrocaffeic acid was chosen as the appropriate substrate in terms of higher bioconversion yield and antioxidant activity. Chapter VI involves the optimization of the enzymatic synthesis of potential phenolic lipids from fish liver oil and dihydrocaffeic acid in solvent-free medium, using response surface methodology. Selected parameters affecting the enzymatic synthesis of phenolic lipids were studied. Also the effects of selected factors limiting the bioconversion yield and productivity were investigated aiming to scale up the bioprocess.

CHAPTER VI

OPTIMIZATION OF LIPASE-CATALYZED TRANSESTERIFICATION OF FISH LIVER OIL AND DIHYDROCAFFEIC ACID IN SOLVENT-FREE MEDIUM USING RESPONSE SURFACE METHODOLOGY

6.1. Abstract

The optimization of the enzymatic synthesis of phenolic lipids from fish liver oil and dihydrocaffeic acid (DHCA) in solvent-free medium (SFM) was carried out, using response surface methodology (RSM). Selected reaction parameters involved in the enzymatic synthesis of phenolic lipids, including temperature, agitation speed, enzyme and phenolic acid concentrations, were investigated. In addition, the effects of water activity and Silica gel on the bioconversion yield and productivity were determined. Statistical analysis showed that phenolic acid and enzyme concentrations as well as agitation speed were the most important factors, that exert a statistically significant overall effect on the phenolic lipids bioconversion yield. The optimal conditions for the enzymatic reaction were obtained at temperature 50.0ºC, with 20.9 mM phenolic acid, enzyme concentration 51.2 mg of solid enzyme (512 PLU)/mL reaction, agitation speed of 160 rpm, water activity of 0.50 and 3.45 mg Silica gel/mL. The bioconversion yield, obtained under these conditions, was 86.5%, which was very close to the predicted value of 84.5%. The predicted values confirmed the validation of the experimental values. The overall results demonstrated that RSM could be applied effectively to optimize lipase-catalyzed synthesis of phenolic lipids in SFM using fish liver oil and DHCA.

6.2. Introduction

The importance of marine oils in human nutrition and disease prevention is well recognized (Simopoulos, 1991; Riediger *et al*., 2009). Fish oil is one of the main dietary sources of ω-3 long-chain polyunsaturated fatty acids (LC PUFAs), including linolenic acid (LA, $C_{18:3}$ *n*-3) and its longer-chain metabolites, eicosapentaenoic acid (EPA, $C_{20:5}$ *n*-3) and docosahexaenoic acid (DHA, $C_{22:6}$ *n*-3). Beneficial health effects of ω-3 PUFA, especially EPA and DHA, in the prevention of cardiovascular diseases, autoimmune diseases, arthritis and cancers have been widely proven (Larsson *et al.,* 2004; Jung *et al*., 2008; Riediger *et al.,* 2009). Oily fish and fish liver oil supplements being the predominant dietary sources of ω-3 LC PUFAs are prone to

oxidative deterioration (Ito *et al*., 1986). The incorporation of phenolic acids into fish liver oil by lipase-catalyzed transesterification could potentially result in novel molecules, with numerous combined beneficial properties from both molecules (Sabally *et al*., 2007).

Many reaction parameters can affect synergistically and/or antagonistically the bioconversion yield of phenolic lipids. The optimization of the reaction parameters, involved in the enzymatic synthesis of phenolic lipids, is commonly carried out by varying one parameter at a time, while keeping the others constant (Lue *et al*., 2005; Sabally *et al.,* 2006a,b and 2007; Karboune *et al.,* 2008; Karam *et al*., 2009). However, in order to demonstrate the relationship between the variables and the response when there is interaction between them, the response surface methodology (RSM) could be an effective statistical technique (Rodrigues *et al.,* 2008). RSM is used in the investigation of complex processes and optimization of multiple variables to predict the appropriate conditions, using minimum number of experiments (Mutalik *et al.,* 2008; Rodrigues *et al.,* 2008). RSM has several advantages when it is compared, in terms of time and cost, to the classical optimization method, as it determines the interaction effects of multiple parameters on the response (Myers *et al*., 2009). The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. Recently, optimization of lipase-catalyzed synthesis of various esters by RSM has been investigated (Macedo *et al*., 2004; Keng *et al*., 2005; Senanayake and Shahidi, 2006; Zheng *et al*., 2009).

The presented work is part of an on-going research in our laboratory for the enzymatic synthesis of selected phenolic lipids with enhanced anti-oxidative and solubility properties (Lue *et al*., 2005; Safari *et al*., 2006; Sabally *et al.,* 2006a,b; Sabally *et al.,* 2007; Karboune *et al.,* 2008; Karam *et al.,* 2009). Previous work in our laboratory has shown the feasibility of the biotechnological approach for the enzymatic synthesis of phenolic lipids in SFM, using flaxseed and fish liver oils with selected phenolic acids (Chapters III, IV and V). The volumetric productivity of phenolic lipids in SFM was increased 11-fold as compared to that in organic solvent medium (OSM) (Chapter III). There was a significant increase of the relative proportions of the two highly desirable essential fatty acids EPA and DHA, in the synthesized phenolic lipids from fish liver oil. In addition, the synthesized phenolic lipids demonstrated radical scavenging

activity comparable to that of α-tocopherol, as well as a higher oxidative stability as compared to the unmodified fish liver oil (Chapter V).

The objective of the present work was to optimize the enzymatic synthesis of phenolic lipids in SFM, from fish liver oil and DHCA, using RSM. Investigating selected reaction parameters, including temperature, agitation speed, phenolic acid and enzyme concentrations, in order to evaluate the relationship among them, and to demonstrate their effects on the bioconversion yield. The effects of water activity and Silica gel on the bioconversion yield and productivity were also investigated.

6.3. Materials and Methods

6.3.1. Materials

Commercial immobilized lipase from *Candida antarctica* (Novozym 435, with an activity of 10,000 propyl laurate units, PLU, per g solid enzyme) was obtained from Novozymes Nordisk A/S (Bagsværd, Denmark). Fish liver oil, dihydrocaffeic acid, Silica gel (60-200 mesh) and salt hydrates pairs, were purchased from Sigma Chemical Co. (St-Louis, MO), including lithium sulfate 1/0 with a_w of 0.19, barium chloride 2/1 with a_w of 0.33, sodium pyrophosphate 10/0 with a_w of 0.67, sodium borate 10/0 with a_w of 0.80, where the salt hydrate pairs are referred to by a shorthand notation giving the number of water molecules. Organic solvents of high-performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific (Fair Lawn, N.J.).

6.3.2. Experimental Design

A four-factor-five-level central composite rotatable design (CCRD) was employed, requiring 30 experiments (Brereton, 2003). The design consists of 16 factorial, 8 axial and 6 center points. An appropriate range for each factor was determined for RSM. The variables and their selected levels for the enzymatic synthesis of phenolic lipids were, temperature (40-60ºC), phenolic acid concentration (10-50 mM), enzyme concentration (30-70 mg of solid enzyme, 300-700 PLU/mL reaction) and agitation speed (50-250 rpm). The design of the employed experiments is presented in Table 13. The phenolic lipids productivity has been investigated using the optimized condition obtained from the first design, with variable Silica gel (1.5-6.6 mg/mL) and water activity (0.19-0.80) that required 13 additional experiments. The experiments were carried out in random order and duplicate measurements of the bioconversion yield percentage were carried out for each

experimental trial. A software package by Design Expert Version 7. (State-Ease Inc., Statistics Made Easy, Minneapolis, MN) was used to fit the second-order model to the independent variables (Myers *et al*., 2009) using the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad \text{(Equation 1)}$$

Where, Y is the dependent variable (percentage bioconversion yield) to be modeled, x_i and x_j are the independent variables (factors), while $ß_0$, $ß_i$, $ß_{ii}$ and $ß_{ij}$ are the regression coefficients of the model. An analysis of variance (ANOVA) was used to determine whether the constructed model was adequate to describe the observed data. The coefficient of determination (R^2) indicates the percentage of the variability of the optimization parameters that is explained by the model. Three-dimensional surface and contour plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones.

6.3.3. Transesterification Reaction in Solvent-Free Medium

Lipase-catalyzed transesterification of fish liver oil with DHCA in SFM was carried out according to the modified method of Sabally *et al*. (2006b), developed in our laboratory, as described in Chapter III. Prior to each enzymatic reaction, a stock solution of phenolic acid (143-715 mM) in 2-butanone was freshly prepared. Aliquot of DHCA stock solution was mixed with the fish liver oil to acquire a final concentration of 10 to 50 mM, where 2-butanone was 7% of the total reaction volume. Enzymatic reaction was initiated by the addition of lipase in the range 30-70 mg of solid enzyme (300 to 700 PLU)/mL reaction. The reaction mixture was incubated with continuous shaking in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) according to the specified temperature (40-60ºC), and agitation speed (50-250 rpm) in the design. The transesterification reactions were run in duplicate in tandem with control trials that contain all components except the enzyme. The enzymatic reaction was monitored at selected time intervals over the course of 7 days period of time. The recovered samples were flushed with a gentle stream of nitrogen and stored at - 80ºC for further analysis.

6.3.4. Characterization of End Products

6.3.4.1. HPLC Analysis of the Reaction Components

HPLC analysis of reaction mixtures was carried out according to the method developed in our laboratory (Sabally *et al.,* 2007). The separation was performed on an Agilent Zobrax SB-C18 reversed-phase column (250x4.6 mm, 5 µm), using a Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). A volume of 20 µL sample was recovered from the reaction mixture and solubilized in 150 µL isopropanol. A volume of 20 µL of the diluted sample was subjected to HPLC analysis. The elution of the injected sample was carried out by a gradient solvent system, using methanol as solvent (A) and isopropanol as solvent (B). The elution was initiated by an isocratic flow of 100% of solvent A for 10 min, followed by a 10 min linear gradient to 40 and 60% of solvent A and B, respectively, then to 100% of solvent B for 10 min period. The elution was maintained for an additional period of 5 min before reverting to the initial conditions (100% solvent A), followed with an equilibration period of 10 min for the next sample. The flow rate was at 1 mL/min and the detection was performed at 280 nm for monitoring phenolic lipids products. The maximal bioconversion yield of phenolic lipids was calculated as the total peak area of phenolic lipids, detected at 280 nm, divided by the peak area of the residual phenolic acid and that of total phenolic lipids, multiplied by 100.

6.4. Results and Discussion

6.4.1. Model Fitting and ANOVA

Response surface methodology consists of an empirical modelization technique, which has been used to evaluate the relation between predicted and experimental results (Myers *et al*., 2009). Central composite rotatable design (CCRD) is generally considered as the most appropriate design for response surface optimization (Jeong and Park, 2006). CCRD was used throughout this study to provide a proper model for the optimization of the enzymatic synthesis of phenolic lipids in SFM from fish liver oil and DHCA, with four factors and five levels, including reaction temperature, agitation speed, phenolic acid and enzyme concentrations. The experimental actual and coded data are given in Tables 13 and 14. The predicted values were obtained by a model fitting technique using the software Design Expert Version 7 and were seen to be sufficiently

correlated with the experimental ones. The best-fitting model was determined by multiple regression analysis. Fitting the data to various models, linear, two factorial, quadratic and cubic as well as their subsequent ANOVA showed that the transesterification of fish liver oil and DHCA in SFM was most suitably described with a quadratic polynomial model as follows:

Bioconversion Yield (%) = + 61.08 + 0.95A – 6.60B + 2.40C+1.60D + 1.08AC + 1.01BC + 1.88 CD – $4.93A^2$ – $3.91B^2$ – $3.36C^2$ – $4.88D^2$. (Equation 2)

Where, A is the temperature, B is the phenolic acid concentration, C is the enzyme concentration and D is the agitation speed.

The ANOVA for the RSM is shown in Table 15. Model coefficients, F- and P-values, were generated by the software and can be used to evaluate the model (Zheng *et al.,* 2009). The computed F-value (85.83) of the model is high as compared with the tabular value $F_{0.05\ (11,\ 18)}$ 2.37, implying that the model is significant at the 5% significance level. The pure error was very low, indicating good reproducibility of the experimental data. The very low P-value (0.0001) and the appropriate coefficient of determination (R^2 = 0.98) indicate that the quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response and the significant variables. "P-values" less than 0.05 indicate that model terms are significant. In this case, A, B, C, D, A^2, B^2, C^2, D^2, AC, BC and CD are significant model terms, while AB, AD and BD were not significant and can be eliminated. According to the ANOVA of factors, the 'lack of fit F-value' of 1.99 was lower than the tabulated one $F_{0.05\ (13,\ 5)}$ 4.66, implying hence that the lack of fit was not significant relatively to the pure error. These results indicate that the model represents the relationships of reaction parameters well within the selected ranges.

The results (Equation 2) revealed that the phenolic acid concentration was the most significant factor as shown from the regression coefficient ($ß_2$= -6.60), followed in order, by the enzyme concentration ($ß_3$= 2.40), the agitation speed ($ß_4$= 1.60) and the temperature ($ß_1$= 0.95), exerting a statistically significant overall effect (*P*-value < 0.05) on the response. In addition, the coefficients of all interactive terms (AC, BC and CD) had positive signs. A positive sign represents a synergistic effect, whereas a negative one indicates an antagonistic effect.

Table 13. Process variables and their levels used in Central Composite Rotatable Design (CCRD).

		Coded levels				
Variable	Name	-2	-1	0	1	2
A	Temperature (ºC)	40	45	50	55	60
B	Phenolic acid concentration (mM)	10	20	30	40	50
C	Enzyme concentration (mg/mL)	30	40	50	60	70
D	Agitation speed (rpm)	50	100	150	200	250

Table 14. Central composite rotatable quadratic polynomial model, experimental data, observed and predicted values for four-factor-five-level response.

Run no.	Temperature (ºC)	Phenolic acid (mM)	Enzyme conc. (mg/mL)	Agitation speed (rpm)	Observed yield[a] (%)	Predicted yield (%)
1	45	20	40	100	50.0	49.6
2	55	20	40	100	47.7	49.4
3	45	40	40	100	32.0	34.4
4	55	40	40	100	34.0	34.1
5	45	20	60	100	47.0	46.5
6	55	20	60	100	51.5	50.5
7	45	40	60	100	34.2	35.3
8	55	40	60	100	40.7	39.4
9	45	20	40	200	46.6	49.1
10	55	20	40	200	48.0	48.8
11	45	40	40	200	34.5	33.8
12	55	40	40	200	33.0	33.6
13	45	20	60	200	53.5	53.4
14	55	20	60	200	57.0	57.5
15	45	40	60	200	44.5	42.3
16	55	40	60	200	46.9	46.3
17	40	30	50	150	40.1	39.4
18	60	30	50	150	43.3	43.3
19	50	10	50	150	60.0	58.6
20	50	50	50	150	31.6	32.3
21	50	30	30	150	46.0	42.9

[a]Bioconversion yield % was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

Table 14. (Continued). Central composite rotatable quadratic polynomial model, experimental data, observed and predicted values for four-factor-five-level response.

Run no.	Temperature (ºC)	Phenolic acid (mM)	Enzyme conc. (mg/mL)	Agitation speed (rpm)	Observed yield[a] (%)	Predicted yield (%)
22	50	30	70	150	50.0	52.4
23	50	30	50	50	39.0	38.3
24	50	30	50	250	44.8	44.8
25	50	30	50	150	60.5	61.1
26	50	30	50	150	62.0	61.1
27	50	30	50	150	60.5	61.1
28	50	30	50	150	62.0	61.1
29	50	30	50	150	59.0	61.1
30	50	30	50	150	62.5	61.1

[a]Bioconversion yield % was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

Table 15. Analysis of variance (ANOVA) and model coefficients of the first response surface methodology design.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	2815.44	11	255.95	85.83	< 0.0001[a]
A	21.85	1	21.85	7.33	0.0144[a]
B	1044.12	1	1044.12	350.15	< 0.0001[a]
C	137.76	1	137.76	46.20	< 0.0001[a]
D	61.76	1	61.76	20.71	0.0002[a]
A^2	667.83	1	667.83	223.96	< 0.0001[a]
B^2	419.20	1	419.20	140.58	< 0.0001[a]
C^2	309.54	1	309.54	103.81	< 0.0001[a]
D^2	654.37	1	654.37	219.45	< 0.0001[a]
AC	18.71	1	18.71	6.27	0.0221[a]
BC	16.20	1	16.20	5.43	0.0316[a]
CD	56.63	1	56.63	18.99	0.0004[a]
Residual	53.67	18	2.98		
Lack of Fit	44.97	13	3.46	1.99	0.2314[b]
Pure Error	8.71	5	1.74		
Cor Total	2869.11	29			
R-Squared	0.97				
Std. Dev.	1.73				

[a]Significant at *p*-value less than 0.05.
[b]Not significant at *p*-value higher than 0.05.

6.4.2. Effects of Selected Parameters on the Enzymatic Synthesis of Phenolic Lipids

The enzymatic synthesis of phenolic lipids in SFM by the transesterification of fish liver oil with DHCA was investigated, using Novozym 435 from *Candida antarctica* as the biocatalyst (Fig. 19). The effects of reaction temperature, enzyme concentration and their interactive effects on phenolic lipids bioconversion yield are shown in three-dimensional surface plot (Fig. 24). The phenolic acid concentration (30 mM) and agitation speed (150 rpm) were maintained constant at their center points. The analysis of the response surface plots (Table 15) show that the interaction between the enzyme concentration and reaction temperature was significant.

The reaction temperature plays an important role in enzyme activation/deactivation and thus strongly affects the bioconversion yield (Biselli *et al.,* 2002). Although an increase in the reaction temperature enhances its rate, the enzyme becomes more susceptible to thermal deactivation (Biselli *et al.,* 2002). The results (Fig. 24) show that the bioconversion yield was increased from 40.1 to 62.5%, with a concomitant increase in temperature up to 52ºC, and thereafter decreased to 43.3% with further increase to 60ºC. Increasing the temperature may promote the collision between the enzyme and the substrate molecules, which may result in accelerated rates of the enzymatic reaction up to a limit, followed by a decrease thereafter (Biselli *et al.,* 2002). Higher reaction temperatures tend to induce enzyme inactivation which may be due to denaturation process. Arcos *et al*. (1998) reported that the enzymatic synthesis of acylglycerols in SFM from linolenic and glycerol by Novozym 435 was increased with the increase in temperature in the range of 30 to 70ºC; however, the deactivation of the enzyme occurred when the temperature was set at 70ºC. Lue *et al*. (2005) reported that the highest enzyme activity of Novozym 435 was obtained at 55°C for the enzymatic synthesis of phenolic lipids in OSM using cinnamic acid and oleyl alcohol. The experimental findings (Fig. 24) are similar to those reported in literature (Jeong and Park, 2006) for Novozym 435, where the optimum temperature was obtained in the range of 40 to 60ºC. The experimental results (Fig. 24) show that a reaction temperature between 50-55ºC favored the maximal bioconversion yield. Guo and Sun (2007) reported that the enzymatic synthesis of 1,3-diglyceride of linolenic acid in SFM using Novozym 435, resulted in a bioconversion yield of 92-96% at 45 to 55°C. Similarly, Köse *et al*. (2002) and Azócar *et al.* (2010) reported that using Novozym 435 for the enzymatic synthesis of fatty acid methyl ester, by alcoholsis of rapeseed and cotton seed oils, was enhanced

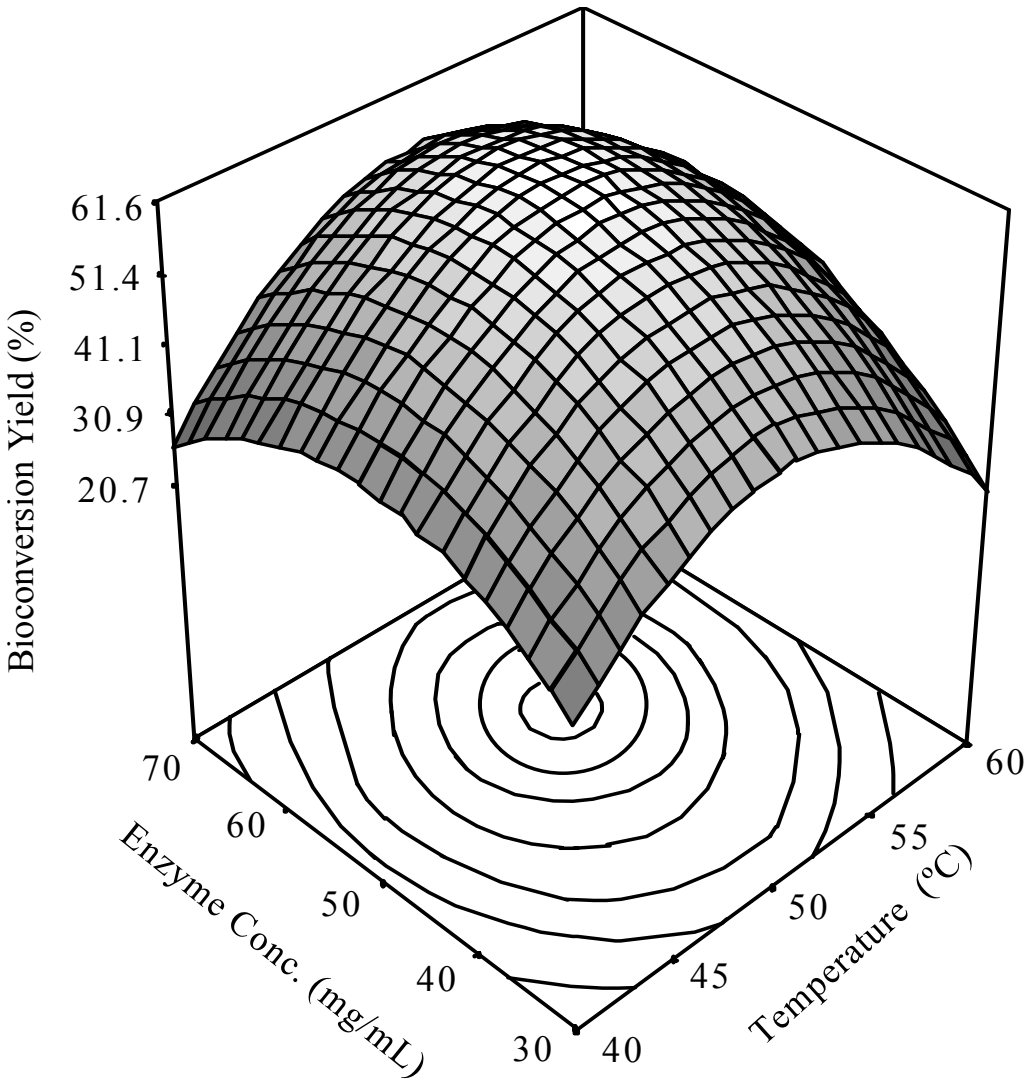


Figure 24. Response surface plot showing the effect of temperature, enzyme concentration and their interaction on the enzymatic synthesis of phenolic lipids, other variables are constant, 30 mM phenolic acid and agitation speed 150 rpm.

when the reaction temperature was adjusted at 45 to 50°C; however, the synthesis was decreased at temperatures higher than 50°C, which could be a result of the enzyme deactivation. In contrast, Zheng *et al.* (2009) reported that higher reaction temperature (65ºC) favored the maximal bioconversion yield of phenolic lipids by Novozym 435 in SFM using ethyl ferulate and triolein as substrates. In addition, Sun *et al.* (2009) reported that feruloylated diacylglycerols synthesis by the esterification of glyceryl ferulate and oleic acid in SFM was obtained at 65°C, without any thermal deactivation of Novozym 435.

The results (Fig. 24) show that at the lowest level of reaction temperature (40°C), the bioconversion yield was increased with a concomitant increase in the enzyme concentration up to 60 mg of solid enzyme (600 PLU)/mL; however, further increase in the enzyme concentration resulted in a decrease in the bioconversion yield. This decrease could be attributed to the fact that there was great resistance of mass transfer with high enzyme loading when the temperature was low in SFM (Zheng *et al.*, 2009). On the other hand, at the highest level of reaction temperature (60°C) the bioconversion yield was increased to a certain extent before its decrease when the enzyme concentration was raised from 50 to 70 mg of solid enzyme/mL. Higher temperatures may increase the solubility of the phenolic acid and also decrease the viscosity of the mixture at longer reaction times, resulting in an enhancement of the bioconversion yield to a certain point before its decrease thereafter as a result of enzyme denaturation (Zheng *et al.*, 2009). With reference to the F-value (7.32) and P-value (0.01), the effect of temperature was less significant than that of the enzyme concentration, with F-value (46.20) and P-value (< 0.0001) in the model (Table 15); however, there was an interaction between the two parameters with P-value (0.0221) as indicated from the model (Table 15). On the other hand, increasing the enzyme concentration from 30 to 50 mg of solid enzyme/mL resulted in an increase of bioconversion yield from 46.0 to 62.5%. However, further increase in the enzyme concentration to 70 mg of solid enzyme/mL resulted in a decrease in the bioconversion yield to 50% (Fig. 24). These results are likely due to mass transfer limitation at a high enzyme concentration, which could have affected the diffusion of the substrate to the enzyme active site and hence its availability for the reaction (Palmer, 1995; Dossat *et al.,* 1999). The decrease in the bioconversion yield may also be explained by the increase in protein-protein interactions at higher enzyme concentration, which could have altered its active conformation and hence its catalytic efficiency (Yadav and Lathi, 2003). In addition,

this decrease may be due to a shift in the reaction thermodynamic equilibrium towards hydrolysis (Karboune *et al*., 2005).

Karam *et al*. (2009) reported that the highest bioconversion yield (83%) of phenolic lipids was obtained with 50 mg of solid enzyme (500 PLU) at 55ºC, using fish liver oil and 3,4-dihydroxyphenyl acetic acid (DHPA) as substrates in OSM; however, increasing the enzyme concentration to 60 mg of solid enzyme (600 PLU) decreased the bioconversion yield to 60%. In addition, Awang *et al.* (2004) reported that an excess of Novozym 435 (> 0.2 g) did not increase the esterification yield of oleic acid with oleyl alcohol. At high enzyme concentration, a decrease in its activity may occur due to the steric hindrance of its active site (Hadzir *et al.*, 2001). Likewise, Senanayake and Shahidi (2002) indicated that the extent of docosahexaenoic acid incorporation into borage oil was increased by increasing the amount of the enzyme in the mixture; however, there was no significant increase in the bioconversion when the amount of enzyme was greater than 100 units. Zheng *et al.* (2009) reported that 50 mg of Novozym 435 (500 PLU)/mL favored a maximum bioconversion yield of phenolic lipids in SFM. Similar results were obtained (Chapter IV) for the transesterification of flaxseed oil with DHPA in SFM, using Novozym 435, where the enzymatic activity was significantly decreased from 178 to 100 nmol of PLs/g solid enzyme/min when the enzyme concentration was increased from 40 to 70 mg of solid enzyme/mL.

The effects of phenolic acid and enzyme concentrations and their interactive effects on phenolic lipids bioconversion yield at temperature 50ºC and agitation speed 150 rpm are shown in Figure 25. The bioconversion yield decreased when the phenolic acid concentration was increased, as indicated by the negative sign in equation 2. The bioconversion yield remained relatively constant (60.0 to 62.5%) when DHCA concentration was increased from 10 to 30 mM, before its decrease to 57.2% when DHCA was 34.7 mM; however, the bioconversion yield decreased significantly to 31.6 % when DHCA was increased to 50 mM. This decrease may be attributed to the enzyme inhibition by the excess of the phenolic acid. Similarly, Lee and Akoh (1998) reported that increasing caprylic acid concentration in the transesterification reaction of peanut oil and caprylic acid resulted in an inhibition in the lipozyme activity. Likewise, our results (Chapter III) indicated that increasing DHPA concentration from 20 to 60 mM resulted in a 10% decrease in the bioconversion yield of phenolic lipids in SFM, using flaxseed oil and DHPA.

The presence of larger amounts of substrates generally increases the probability of substrate-enzyme collision, and hence the increase in the amount of enzyme could lead to an increase in the bioconversion yield. This relationship is true when there are no limiting factors, such as low substrate concentration, presence of activators/inhibitors or mass transfer effect (Gunawan *et al*., 2005).

Among the three interactions, which had great effect on phenolic lipids bioconversion yield, is the interaction between the enzyme concentration and agitation speed (Equation 2). The effects of enzyme concentration, agitation speed and their interactive effects on phenolic lipids bioconversion yield, at 30 mM phenolic acid and reaction temperature 50ºC are shown in Figure 26. The bioconversion yield increased significantly from 39.0 to 62.5% when the agitation speed was increased from 50 rpm to 150 rpm, before its decrease to 44.8% at agitation speed 250 rpm. The low bioconversion yield could be attributed to insufficient agitation rate, a condition in which a hydrophilic layer of glycerol may be formed around the enzyme, limiting hence the mass transfer rate of the oil to the surface of the lipase (Dossat *et al.,* 1999). Soumanou and Bornscheuer (2003) reported a low bioconversion yield of alcoholysis in SFM, which was attributed to the unfavourable viscosity conditions that may have affected the mixing of the substrates with the lipase. The increase in the rate of agitation may decrease the boundary liquid layer surrounding the porous support, resulting in lower diffusional limitations (Barros *et al*., 1998). Hadzir *et al*. (2001) reported that an agitation speed of 150 rpm was selected for the enzymatic alcoholysis of triolein by Novozym 435. Similar results were also obtained by Lue *et al.* (2005) for the enzymatic synthesis of phenolic lipids in OSM using cinnamic acid and oleyl alcohol, as well as for the transesterification of flaxseed oil with DHPA in SFM by Novozym 435, where the optimum agitation speed for the enzymatic synthesis of phenolic lipids was set at 150 rpm (Chapter IV).

The experimental results (Table 16) show that, even after the optimization of the four selected parameters in the design, there was an overall limited increase in the bioconversion yield. This could be explained by an increase in the concentrations of glycerol and or/free fatty acids as by-products of the hydrolysis of triacylglycerols, or may be due to the fact that the reaction reached its equilibrium (Dossat *et al.,* 1999; Karboune *et al.,* 2005).

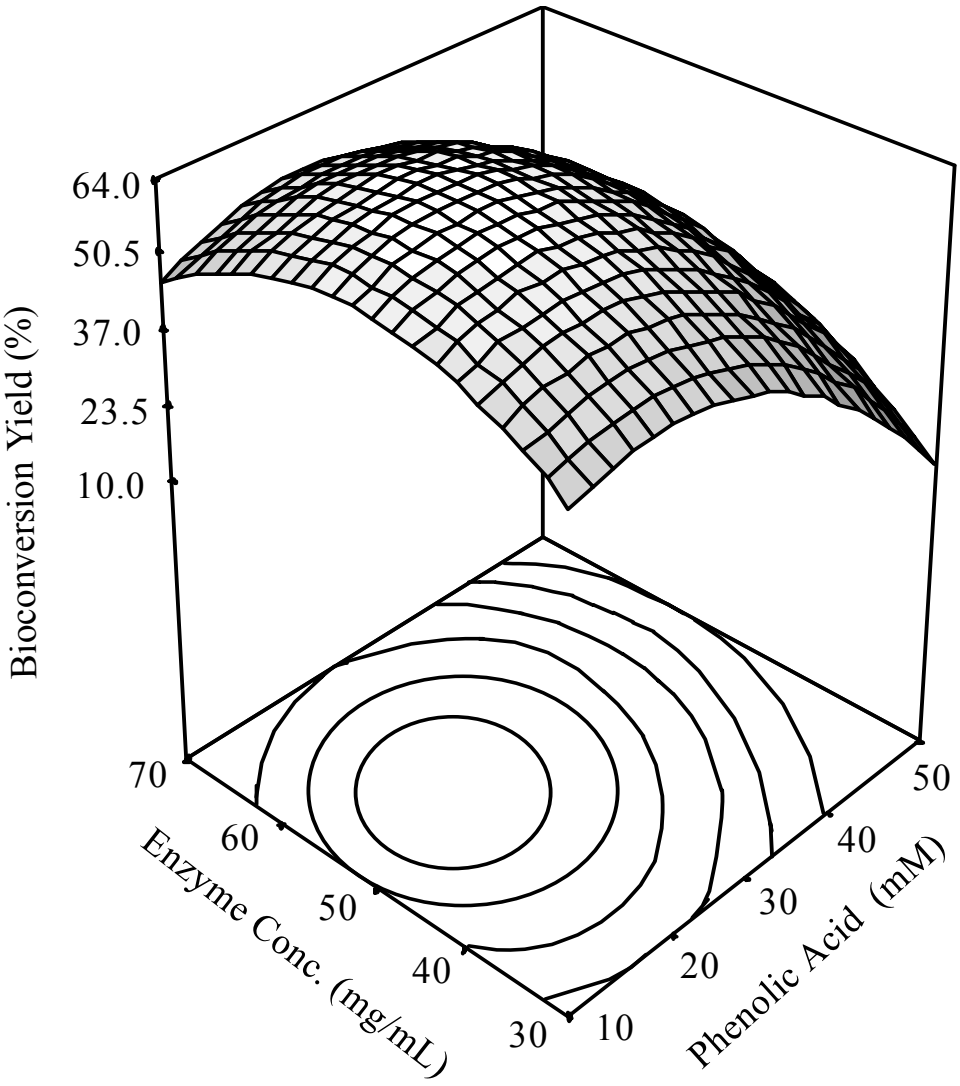


Figure 25. Response surface plot showing the effect of phenolic acid concentration, enzyme concentration and their interaction on the enzymatic synthesis of phenolic lipids, other variables are constant, temperature 50°C and agitation speed 150 rpm.

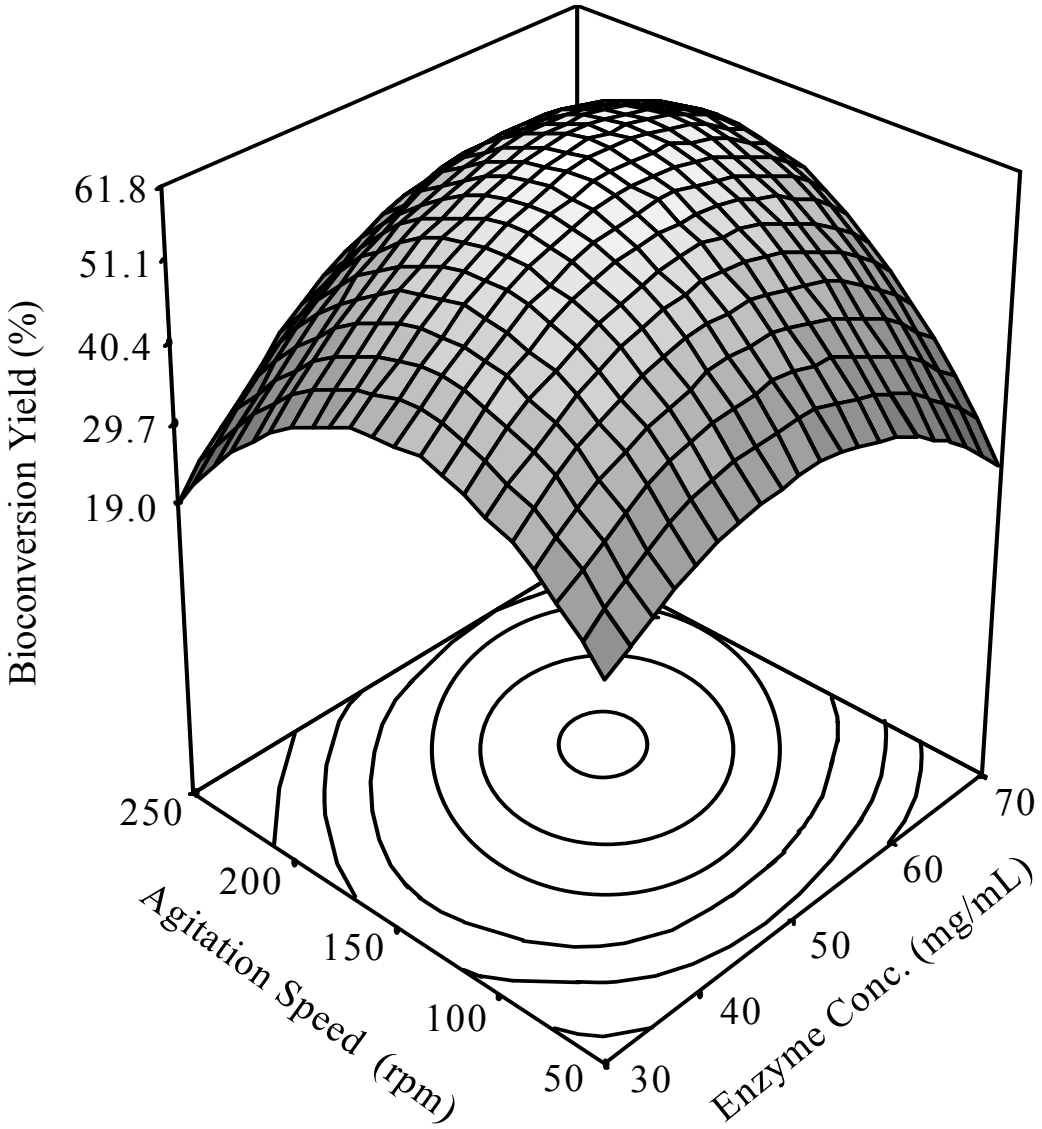


Figure 26. Response surface plot showing the effect of enzyme concentration, agitation speed and their interaction on the enzymatic synthesis of phenolic lipids, other variables are constant, 30 mM phenolic acid and temperature 50°C.

The accumulation of glycerol molecules in the enzyme micro-environment may lead to the formation of the hydrophilic hindrance layer, limiting the substrate diffusion and hence the bioconversion yield (Dossat *et al.,* 1999). Similarly, Karboune *et al.* (2005) reported that the formation of a hydrophilic hindrance layer of glycerol around the enzyme molecule could be a valid explanation for the limited increase in the bioconversion yield of cinnamoylated lipids in OSM. The overall experimental data (Table 16) indicates that the maximal bioconversion yield (64.1%) was favored as predicted in the model, using a reaction temperature of 50.0ºC, 20.9 mM phenolic acid, 51.2 mg of solid enzyme (512 PLU)/mL and agitation speed of 160 rpm.

6.4.3. Optimization of the Transesterification Reaction

In order to maximize the bioconversion yield of phenolic lipids, selected limiting factors, including the effects of water activity (a_w) and Silica gel, used as an adsorbent for the removal of the hydrophilic hindrance layer of glycerol surrounding the enzyme support, were investigated. The reaction was conducted under the optimal conditions as predicted in the first designed experiments (Table 16). Silica gel was added to the reaction medium in the range of 1.5-6.0 mg/mL and water activity was varied in the range of 0.19-0.80, using different salt hydrate pairs (Halling, 1992). The experimental design required 13 additional experiments using two-factor-five-level central composite design (Table 17), with 4 factorial, 4 axial and 5 center points.

The ANOVA for the RSM is shown in Table 18. The high computed F-value of 159.8 as compared to the tabulated one $F_{0.05\ (4,\ 8)}$ 3.84 indicates that the model was significant at the 5% significance level. The very small P-value of 0.0001 and a suitable coefficient of determination ($R^2 = 0.98$) indicate that the quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response and the significant variables. "P-values" less than 0.05 indicate that the model terms were significant. In this case, A, B, A^2, B^2 are significant model terms. According to the ANOVA of factors, the 'lack of fit F-value' of 3.6 was low as compared to the tabulated one $F_{0.05\ (4,\ 4)}$ 6.39 which implies that the lack of fit was not significant as relative to the pure error. These results indicate that the model represents the relationships of reaction parameters well within the selected ranges.

Table 16. Solutions predicted for optimization of phenolic lipids synthesis from fish liver oil using response surface methodology.

Solution	Temperature (ºC)	Phenolic acid (mM)	Enzyme concentration (mg/mL)	Agitation speed (rpm)	Bioconversion yield (%)
1	48.9	22.6	51.7	147.4	63.4
2	50.0	20.9	51.2	159.5	64.1
3	52.8	23.6	49.3	162.8	62.5
4	50.8	26.3	51.3	164.7	63.4
5	50.4	21.3	54.9	149.2	63.8
6	49.6	21.5	51.3	151.3	63.9
7	48.9	16.4	51.5	161.2	62.6

Table 17. Central composite quadratic polynomial model, experimental data, observed and predicted values for two-factor-five-level response.

Run no.	Silica gel (mg/mL)	Water activity	Observed yield[a] (%)	Observed P_V[b] (mgPLs/mL reaction/day)	Predicted yield[a] (%)	Predicted P_V[b] (mgPLs/mL reaction/day)
1	2.50	0.33	80.0	1.16	81.4	1.19
2	5.00	0.33	74.7	1.09	76.6	1.11
3	2.50	0.67	66.8	0.97	67.6	0.98
4	5.00	0.67	61.0	0.88	62.7	0.91
5	1.50	0.50	73.0	1.06	72.5	1.05
6	6.00	0.50	65.0	0.95	63.7	0.93
7	3.75	0.19	74.7	1.09	73.5	1.07
8	3.75	0.80	50.4	0.73	49.7	0.72
9	3.75	0.50	85.3	1.24	84.3	1.23
10	3.75	0.50	84.8	1.23	84.3	1.23
11	3.75	0.50	83.0	1.20	84.3	1.23
12	3.75	0.50	85.5	1.24	84.3	1.23
13	3.75	0.50	84.9	1.23	84.3	1.23

[a]Bioconversion yield % was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b]Volumetric productivity (Pv) was calculated on the basis of weight of phenolic lipids in mg per mL reaction mixture per day.

Table 18. Analysis of variance (ANOVA) and model coefficients of the second response surface methodology design[a].

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	1451.13	4	362.78	159.80	< 0.0001[b]
A	62.05	1	62.05	27.33	0.0008[b]
B	498.34	1	498.34	219.51	< 0.0001[b]
A^2	382.39	1	382.39	168.44	< 0.0001[b]
B^2	758.84	1	758.84	334.26	< 0.0001[b]
Residual	18.16	8	2.27		
Lack of Fit	14.22	4	3.56	3.61	0.1208[c]
Pure Error	3.94	4	0.99		
Cor Total	1469.29	12			
R-Squared	0.98				
Std. Dev.	1.5				

[a]The phenolic lipids bioconversion yield has been investigated using the optimized condition obtained from the first design, with variable Silica gel (1.5-6.6 mg/mL) and water activity (0.19-0.80), and it was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b]Significant at *p*-value less than 0.05.

[c]Not significant at *p*-value higher than 0.05.

The investigated factors were most suitably described, with a quadratic polynomial model as follows:

Bioconversion Yield (%) = + 84.25 – 2.43 A – 6.94 B – 4.99 A^2 – 7.16 B^2. (Equation 3)

Where, A is the Silica gel concentration and B is the water activity; there was no interaction between them as indicated from the model (Table 18). Both parameters have an antagonistic effect on the response; however, the effect of water activity was more significant than that of the Silica gel.

The productivity of phenolic lipids was determined to assess the reaction efficiency over defined incubation period for the Novozym 435-catalyzed transesterification reaction. The volumetric productivity (P_V) was calculated by the weight of produced phenolic lipids in mg per mL of reaction mixture per day. The effects of Silica gel, water activity and their effect on phenolic lipids bioconversion yield and the Pv, at temperature (50.0ºC), phenolic acid (20.9 mM), enzyme concentration (51.2 mg of solid enzyme (512 PLU)/mL) and agitation speed (160 rpm) are shown in Figure 27. The water activity (a_w) of the reaction medium is an important parameter, since it affects both the enzymatic activity and the reaction equilibrium (Valivety *et al.,* 1992; Yang *et al.,* 2004). The results (Figs. 27A and 27B) show that the bioconversion yield and the Pv were increased from 74.4 to 84.5% and from 1.09 to 1.23 mg PLs/mL of reaction mixture/day, respectively, when the a_w was increased from 0.19 to 0.33; as the a_w was further increased to 0.50, they remained relatively constant (85.5% and 1.24 mg PLs/mL of reaction mixture/day). However, increasing the a_w from 0.50 to 0.67 and 0.80 resulted in a decrease in the bioconversion yield to 70.2 and 50.4%, respectively, while the P_V was decreased by 1.4 and 1.7-fold. The decrease in the bioconversion yield may be due to a shift in the reaction thermodynamic equilibrium towards hydrolysis (Karboune *et al*., 2005). Graille (1999) reported that lipase-catalyzed reactions in SFM require a minimal hydration to maintain the enzyme activity toward ester synthesis. Only a thin monolayer of bound water is needed to maintain the enzyme three dimensional structure needed to retain its catalytic activity (Halling, 2002).

The experimental results (Fig. 27) are in agreement with those reported by Karra-Chaabouni *et al.* (2002) for the synthesis of geranyl butyrate in SFM by an esterase from *Mucor miehei*, where a high bioconversion yield (75%) was obtained at low initial a_w of 0.2 to 0.5 as compared to 13%

at higher initial a_w of 0.5. Similarly, the experimental results (Chapter IV) indicated that increasing the a_w of the reaction medium from 0.18 to 0.38 resulted in a 15% increase in the bioconversion yield of phenolic lipids in SFM, using flaxseed oil and DHPA; however, further increase of a_w from 0.38 to 0.67 resulted in a significant decrease in the maximum bioconversion yield to 28%. In contrast, the optimal a_w of 0.38 is lower than that (0.13) reported for the transesterification of ethyl ferulate and triolein, in SFM (Zheng *et al*., 2009).

To limit the formation of the glycerol hydrophilic hindrance layer in the enzyme micro-environment, the addition of Silica gel in the reaction mixture as an adsorbent of glycerol was investigated. The enzymatic synthesis of phenolic lipids was investigated (Fig. 27) upon the addition of Silica gel at a concentration range of 1.5-6.0 mg/mL. The experimental results (Figs. 27A and 27B) indicate that the addition of 1.5 mg/mL Silica gel increased the bioconversion yield and the P_V from 62.5 to 73.0% and from 0.9 to 1.06 mg PLs/mL of reaction mixture/day, respectively; however, further increase in the Silica gel to 3.75 mg/mL resulted in an increase in the bioconversion yield and the P_V to 85.5% and 1.24 mg PLs/mL of reaction mixture/day, respectively. Nevertheless, the addition of 6.0 mg/mL Silica gel resulted in a decrease in the bioconversion yield and the P_V to 65% and 0.94 mg PLs/mL of reaction mixture/day. These findings could be explained by the dehydrating effect of Silica gel on the enzyme, which may have resulted in its inactivation (Dossat *et al.,* 1999). Similarly, Karboune *et al.* (2005) reported an increase in the bioconversion yield of cinnamoylated lipids in OSM, from 42 to 55%, upon the addition of 2.2 mg Silica gel/mL to the reaction mixture; however, the bioconversion yield was significantly declined to 26% upon an increase in the Silica gel concentration to 4.4 mg/mL. The addition of an appropriate amount of Silica gel can prevent the adsorption of glycerol on the support of immobilized enzyme, and any excess amount of it may cause enzyme dehydration resulting in its inactivation (Dossat *et al.,* 1999).

The overall limited increase in the P_V may be due to the denaturation of the enzyme (Yadav and Lathi, 2003), of both/either reactants and/or products inhibition (Garcia *et al.*, 1999). Such limited increase in the productivity also might be explained by the formation of a hydrophilic hindrance layer of the glycerol in the micro-environment of the enzyme, which can also increase the substrate diffusion limitations (Karboune *et al.*, 2005; Wei *et al*., 2008).

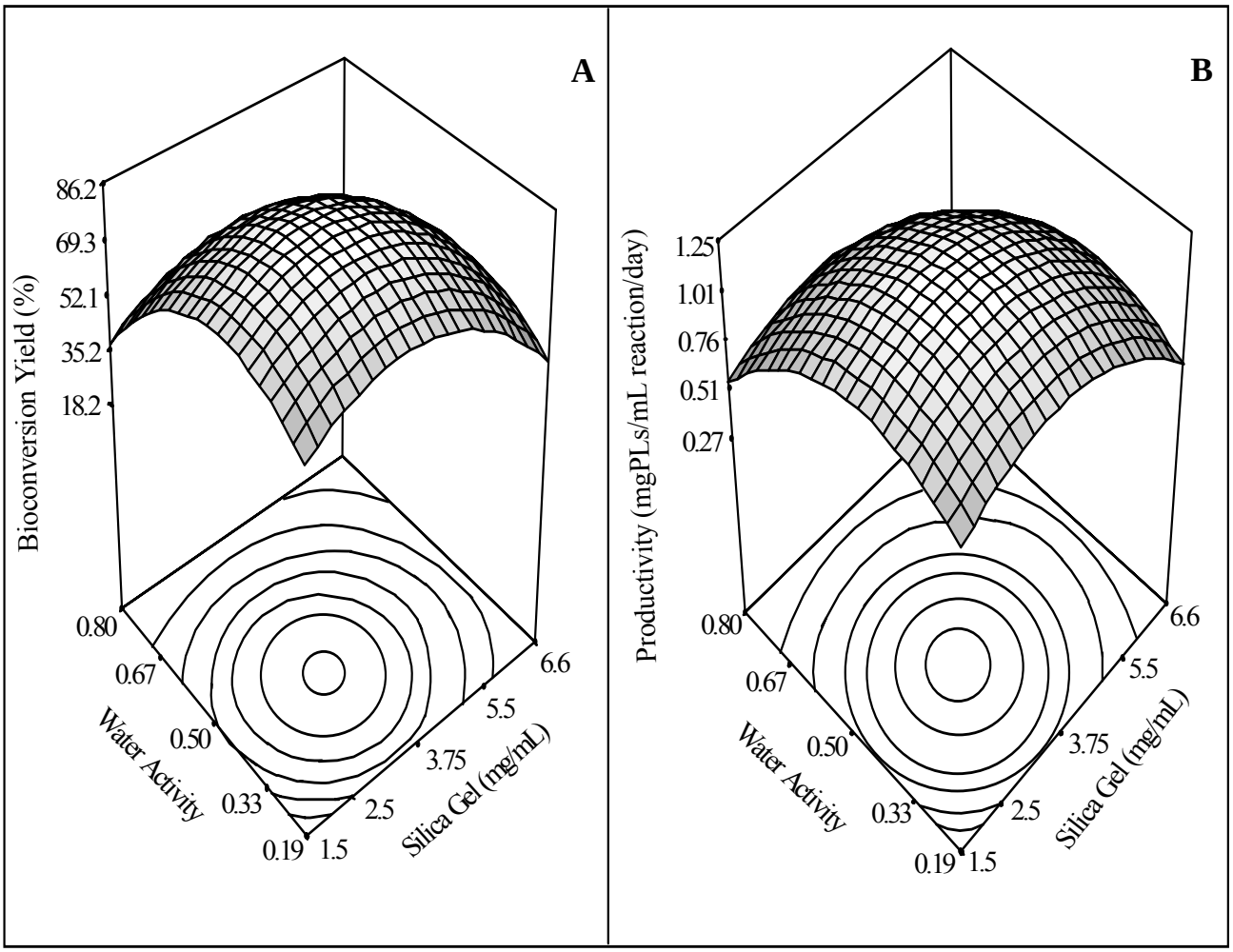


Figure 27. Response surface plot showing the effect of water activity, Silica gel on the bioconversion yield (A) and the volumetric productivity (B) of phenolic lipids.

6.4.4. Optimal Conditions and Model Validation

Response surface methodology can present the optimum combination of parameters that could be selected to obtain the highest bioconversion yield. The optimum conditions for the enzymatic synthesis of phenolic lipids are presented in Table 19, along with their predicted and experimental values. The optimal conditions for the lipase-catalyzed transesterification of fish liver oil and DHCA were a temperature of 50.0ºC, 20.9 mM phenolic acid, 51.2 mg of solid enzyme (512 PLU)/mL, agitation speed of 160 rpm, water activity of 0.50 and 3.45 mg Silica gel/mL.

In order to validate the model, five experiments were carried out under the optimal conditions, as predicted by the model (Table 19). The results show that the maximum bioconversion yield was 86.5% and the predicted one was 84.5%. While the observed and predicted P_V were 1.26 and 1.23 mg PLs/mL of reaction mixture/day, respectively. Chi-square test showed insignificant difference at $P < 0.01$ indicating, hence, a good correlation between the experimental results and the statistical one, as predicted by the model; the overall results hence confirm the validity and adequacy of the predicted model.

The maximum bioconversion yield of dihydrocaffeoylated fish liver oil (86.5%) in SFM is within the range of those reported in OSM (65 and 83%) for the transesterification of fish liver oil with DHCA (Sabally *et al*., 2007) and DHPA (Karam *et al*., 2009). On the other hand, The P_V for the transesterification of DHCA and DHPA with fish liver oil in OSM (Sabally *et al*., 2007; Karam *et al*., 2009) were 0.03 and 0.04 mg PLs/mL of reaction mixture/day, respectively, using 5 mM of phenolic acid at a substrate molar ratio of 1:8. These values were 31 and 38-fold lower than the maximum Pv obtained using 20.9 mM DHCA in SFM. In conclusion, one of the advantages of using a SFM as compared to OSM as a reaction medium is its higher P_V. Likewise, Dossat *et al.* (2002a) reported a 6-fold increase in the P_V of Lipozyme IM-catalyzed transesterification reaction of sunflower oil with butanol in SFM as compared to OSM.

Table 19. Solutions predicted for optimization of phenolic lipids synthesis using response surface methodology (Model verification experiment).

Solution	Silica gel (mg/mL)	Water activity	Predicted BY[a] (%)	Predicted Pv[b] mg PLs/mL reaction/day	Observed BY[c] (%)	Observed Pv[d] mg PLs/mL reaction/day
1	3.56	0.48	85.8	1.25	81.9 (2.8)[e]	1.19
2	3.29	0.48	85.7	1.25	83.4 (1.2)[e]	1.21
3	3.45	0.50	84.5	1.23	86.5 (1.8)[e]	1.26
4	3.45	0.33	84.3	1.24	82.5 (2.9)[e]	1.20
5	3.44	0.36	85.4	1.24	83.9 (3.1)[e]	1.20

[a,c]The model predicted bioconversion yield (BY) % and observed bioconversion yield (BY) %, was calculated as the total peak area of phenolic lipids divided by the peak area of residual phenolic acid and total phenolic lipids, multiplied by 100.

[b,d]The model predicted volumetric productivity (Pv) and the observed volumetric productivity (Pv), was calculated on the basis of weight of phenolic lipids in mg per mL reaction mixture per day.

[e]Data are average of two determinations and the standard deviations were given.

6.5. Conclusion

RSM was successfully used in the optimization of the transesterification of fish liver oil and DHCA in SFM, by *Candida antarctica* lipase. A high bioconversion yield and productivity were obtained under the optimized conditions. The comparison of the predicted and experimental values showed good correlation between them. The results suggest that the empirical model, derived from RSM, could be used adequately to describe the relationship between the reaction parameters, for the enzymatic synthesis of phenolic lipids and the response (bioconversion yield and productivity). This model could be used to predict phenolic lipids bioconversion yield in SFM under any given conditions, within the experimental range tested. The overall experimental results could contribute to the development of biotechnological process for the synthesis of phenolic lipids in SFM with high volumetric productivity, as compared to that in OSM.

GENERAL CONCLUSION

The aim of this study was to investigate a lipase-catalyzed process in solvent-free medium (SFM) for the synthesis of phenolic lipids, using selected edible oils and phenolic acids. A volume efficient bioprocess for the enzymatic synthesis of selected phenolic lipids from flaxseed and fish liver oils with high productivity were achieved, using the immobilized *Candida antarctica* lipase (Novozym 435). The use of such medium favored the synthesis of phenolic diacylglycerols as compared to phenolic monoacylglycerols, indicating the substantial effect of the enzyme micro-environment on the enzyme selectivity.

The effect of the chemical structure of phenolic acids on the transesterification of flaxseed and fish liver oils, with selected phenolic acids in SFM was of less magnitude as compared to that in organic solvent media (OSM). Water activity influenced the enzymatic synthesis of phenolic lipids, using 3,4 dihydroxyphenyl acetic (DHPA) and dihydrocaffeic acid (DHCA) with flaxseed and fish liver oils, respectively. A significant increase in the enzymatic activity of the lipase-catalyzed transesterification of DHPA and DHCA with flaxseed and fish liver oils, respectively, was obtained upon the addition of the non-ionic surfactant Span 65. Overall, the volumetric productivity of the transesterification of flaxseed oil with DHPA in SFM was increased 11-fold as compared to that in OSM.

The transesterification reaction led to a significant increase in the relative proportion of linolenic acid ($C_{18:3}$ *n*-3) in the synthesized phenolic lipids as compared to that in the unmodified flaxseed oil. In addition, a significant increase of the relative proportions of the two highly desirable essential fatty acids, eicosapentaenoic acid ($C_{20:5}$ *n*-3) and docosahexaenoic acid ($C_{22:6}$ *n*-3), was obtained in the synthesized phenolic lipids from fish liver oil and DHCA.

Response surface methodology (RSM) was successfully used to optimize the transesterification of fish liver oil and DHCA in SFM. Statistical analysis showed that the phenolic acid and enzyme concentrations as well as agitation speed were the most important factors, exerting a statistically significant overall effect on the phenolic lipids bioconversion yield. The reaction temperature was optimized and was found to be in the same range as that reported in literature. The addition of Silica gel to the reaction medium resulted in an improvement of the

bioconversion yield of phenolic lipids from fish liver oil and DHCA. A high bioconversion yield and productivity were obtained under the optimized conditions. The comparison of the predicted and experimental values showed good correlation between them, implying that the empirical model derived from RSM can be used adequately to describe the relationship between the reaction parameters and the bioconversion yield of phenolic lipids. Hence, this model could then be used to predict phenolic lipids bioconversion yield in SFM, within the experimental range.

APCI-MS analysis confirmed the formation of six 3,4-dihydroxyphenyl acetoylated and six dihydrocaffeoylated lipids from the transesterification of flaxseed and fish liver oils, using DHPA and DHCA, respectively, as substrates in SFM. The synthesized phenolic lipids demonstrated radical scavenging activity compared to that of α-tocopherol, indicating hence their potential as antioxidants. In addition, the synthesized phenolic lipids demonstrated higher oxidative stability as compared to the unmodified fish liver oil, indicating the beneficial effect of phenolic acid incorporation into the oil. The investigated approach is a promising and environmentally safe process for the production of structured lipids with phenolic acid moiety. The overall experimental results obtained through the present study could contribute to the development and to the use of SFM as a biotechnological method in industrial processes, with high volumetric productivity, and the novel phenolic lipids can be used in nutraceutical applications.

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